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# Tagging flounder *Platichthys flesus* - a test of methodologies and an evaluation of behavioural and physiological effects

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INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR

VANESSA MARIA SILVA NEVES

# TAGGING FLOUNDER *PLATICHTHYS FLESUS* – A TEST OF METHODOLOGIES AND AN EVALUATION OF BEHAVIOURAL AND PHYSIOLOGICAL EFFECTS

Dissertação de Candidatura ao grau de Mestre em Ciências do Mar – Recursos Marinhos submetida ao Instituto de Ciências Biomédicas de Abel Salazar da Universidade do Porto.

Orientadora – Doutora Vânia de Jesus de Paiva Freitas

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Categoria – Diretor

Afiliação – Aquamuseu do Rio Minho

“To accomplish great things, we must not only act,
but also dream; not only plan, but also believe.”
- Anatole France

## | Scientific output

Until the moment, the scientific output of this thesis has resulted in an oral communication in scientific meetings:

Neves V, Ramos S, Antunes C, Freitas V (2017). Effects of artificial tags on survival, growth, condition, and behavior of flounder *Platichthys flesus*. 10th IJUP - Encontro Investigação Jovem da Universidade do Porto, February 2017, Porto, Portugal.

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| Resumo

A marcação de peixes, com o desenvolvimento de marcas eletrónicas, tornou-se uma importante ferramenta para resolver questões relacionadas com a biologia e ecologia dos peixes, como por exemplo padrões migratórios. Todos os estudos de marcação assentam num pressuposto chave: a população marcada é uma amostra representativa da população geral; e este pressuposto é ainda mais importante quando as marcas transmitem informações contínuas sobre a localização dos animais marcados, como é o caso das marcas acústicas. Para recolher dados fidedignos nem a marca nem o procedimento de marcação devem afetar significativamente o comportamento ou fisiologia dos peixes.

A solha (*Platichthys flesus*) é uma espécie marinha caraterizada por um ciclo de vida complexo com transições entre fases de vida e mudanças de habitat a ocorrerem simultaneamente. A marcação desta espécie com marcas acústicas é um método que poderá ajudar a esclarecer os seus movimentos ontogénicos a uma escala de elevada resolução. No entanto, antes de seguir e recolher dados, a partir de um animal no seu ambiente natural, os possíveis efeitos dos procedimentos de marcação devem ser avaliados.

Neste estudo, solhas foram mantidas em cativeiro para avaliar os efeitos das marcações e de todas as manipulações associadas à marcação. Num estudo preliminar, a dose eficaz de anestésico e analgésico e o tempo de indução para manter os peixes sedados durante as marcações foram determinados. Duas experiências a curto prazo (33 dias), com peixes de diferentes dimensões, e três tratamentos cada (um controlo e dois de marcação, externo e implante ou t-bar), foram realizadas para avaliar os efeitos dos diferentes procedimentos de marcação na sobrevivência, condição, crescimento e comportamento das solhas. O desenvolvimento de úlceras na pele no decorrer das experiências foi explorado num estudo patológico da prevalência e distribuição da doença, e um estudo microbiológico foi realizado para aferir as possíveis causas e outros fatores agravantes.

No fim das experiências de marcação foi claro que o procedimento de marcação externa é o mais adequado para marcar esta espécie. A retenção das marcas foi elevada e não houve efeitos significativos no comportamento alimentar ou natatório. Efeitos na sobrevivência, condição e crescimento foram negativos, no entanto é possível que estes tenham sido influenciados por outros fatores e não apenas pelos procedimentos de marcação. O facto de terem sido mantidas em

cativeiro e desenvolvido úlceras pode ter interferido com os efeitos da marcação, exacerbando os efeitos negativos.
Diretrizes para futura implementação de marcação de solhas no campo também são fornecidas.

| Palavras-chave: telemetria; marcas acústicas; peixe plano; anestesia; úlceras; estuário do Douro

| Abstract

Fish tagging, with the development of electronic tags, has become an incredibly powerful tool to resolve fishes' biology and ecology-related questions such as migration patterns. The key assumption of any tagging study, and especially of those that continuously track an individual such as acoustic tags, rely on the tagging population being a representative sample of the general population. To collect unbiased data, the tag and the tagging procedure should not significantly affect fish's physiology or behaviour.

Flounder (*Platichthys flesus*) is a marine fish species with a complex life cycle with transitions of life stages overlapping with changes in habitat. Marking flounder with acoustic tags would be a method to further explore this species' fine scale ontogenic movements. However, before tracking an animal in its natural environment and collecting data, the effects of the tagging procedures must be assessed.

In this study, flounder were kept in captivity to evaluate tagging and associated manipulation effects. In a preliminary study, the effective dosage of anaesthetic and analgesic, and induction time required to maintain fish sedated during the tagging procedures were determined. Two short-term (33 days) experiments, with different sized flounder and three treatments each (a control and two tagging treatments, external mount and implant or t-bar), were performed to evaluate the effects of different tagging procedures on survival, condition, growth and behaviour. The development of skin ulcers in the course of the experiments was explored in a pathology study of disease prevalence and ulcer distribution, and a microbiological study was performed to analyse its possible causes and aggravating factors.

From the tagging experiments it was clear that the external mount procedure was the most suited to mark flounder. Retention was high and there were no significant effects on feeding or swimming behaviour. Effects on survival, condition and growth were negative however these might have been biased by factors other than the tagging procedures. Captivity and the development of ulcerative skin disease might have interfered with the effects of tagging, exacerbating the negative results. Guidelines for future implementation of flounder tagging in the field are also provided.

| Keywords: telemetry; acoustic tags; flatfish; anaesthesia; skin ulcers; Douro estuary

# | List of figures and tables

## 1 | Figures

## 2 | Tables

ANOVA – One-way analysis of variance
BOGA – Biotério dos Organismos Aquáticos
Bt – Biting
C- – Control group without analgesic
C+ – Control group with analgesic
CG – Control Group
CIIMAR – Centro Interdisciplinar de Investigação Marinha e Ambiental
EG – External Group
Exp. – Experiment
F – Infected flounder
HSD – Honestly Significant Difference
ID – Identification
IG – Implant Group
K – Condition Factor
MS-222 – Tricaine methane-sulphonate
N – Sample size
N/S – Not Stated
NA – Not applicable
OIE – World Organisation for Animal Health
ORBEA – Organismo Responsável pelo Bem-Estar Animal
*P. flesus* – *Platichthys flesus*
PIT – Passive Integrated Transporter
PSATs – Pop-up Satellite Archival tags
psu – Practical salinity units
Sc – Scraping
SD – Standard Deviation
SE – Standard Error
SGR – Specific Growth Rate
spp. – Several species
SW – Southwest
SW – Swimming
TG – T-bar group
TL – Total length
Treat. – Treatment group

U – Ulcer

W – Weight

# Chapter 1 | General introduction

## 1.1 | Fish tagging – an overview of techniques and recent advances

Fish marking and tracking is a fundamental tool for fisheries management and research. It allows the researcher to gather knowledge on fish migration and movements as well as on the dynamics of exploited populations which is key to accurately developing management and conservation plans.

It is not clear when fish were tagged for the first time, however in 1653 an early report was published describing how private individuals tied ribbons to the tail of juvenile Atlantic salmon (*Salmo salar*) discovering that the fish returned from the sea to their natal river (Walton & Cotton, 1847; McFarlane *et al.*, 1990). Since the late 1800s many fish tagging experiments have been developed, with an initial focus on salmonids closely followed by flatfish and cod, which were tagged with the Petersen disk as early as 1894 (Petersen, 1986; McFarlane *et al.*, 1990). Around the 1930s, small pelagic species were successfully tagged with metal body cavity tags (Rounsefell & Dahlgren, 1933; McFarlane *et al.*, 1990) and only in the 1950s were larger pelagic fishes successfully tagged with the development of spaghetti loop tags and dart tags (Wilson, 1953; Yamashita & Waldron, 1958; McFarlane *et al.*, 1990).

Before World War I, tagging studies focused primarily on determining fish movements and stock identification, later their focus expanded in order to include information on age and growth and to estimate population size or mortality and survival rates (Ricker, 1956; McFarlane *et al.*, 1990).

There is a multitude of available marks to choose from including, for instance, mutilation (i.e. clipping or punching fins or other body part), dyes, and physical tags. Physical tags can be more or less technologically advanced, including the simpler Petersen discs and T-bars and the more sophisticated Pop-up Satellite Archival tags (PSATs) and acoustic tags (figure 1.1). There is not one perfect tagging method and so the choice of the tag to use is a crucial step when developing a tagging study. The main aspects to consider when choosing a mark or tag are: (1) objectives of the study; (2) effect on survival, behaviour, reproduction and growth; (3) permanency of the mark (durability, longevity and stability); (4) number and size of organisms to be marked; (5) stress of capture, handling and marking of the organisms; (6) skilful personnel and ease of application; (7) cost of purchasing tags and conducting the experiment (McFarlane *et al.*, 1990; Latour, 2005).

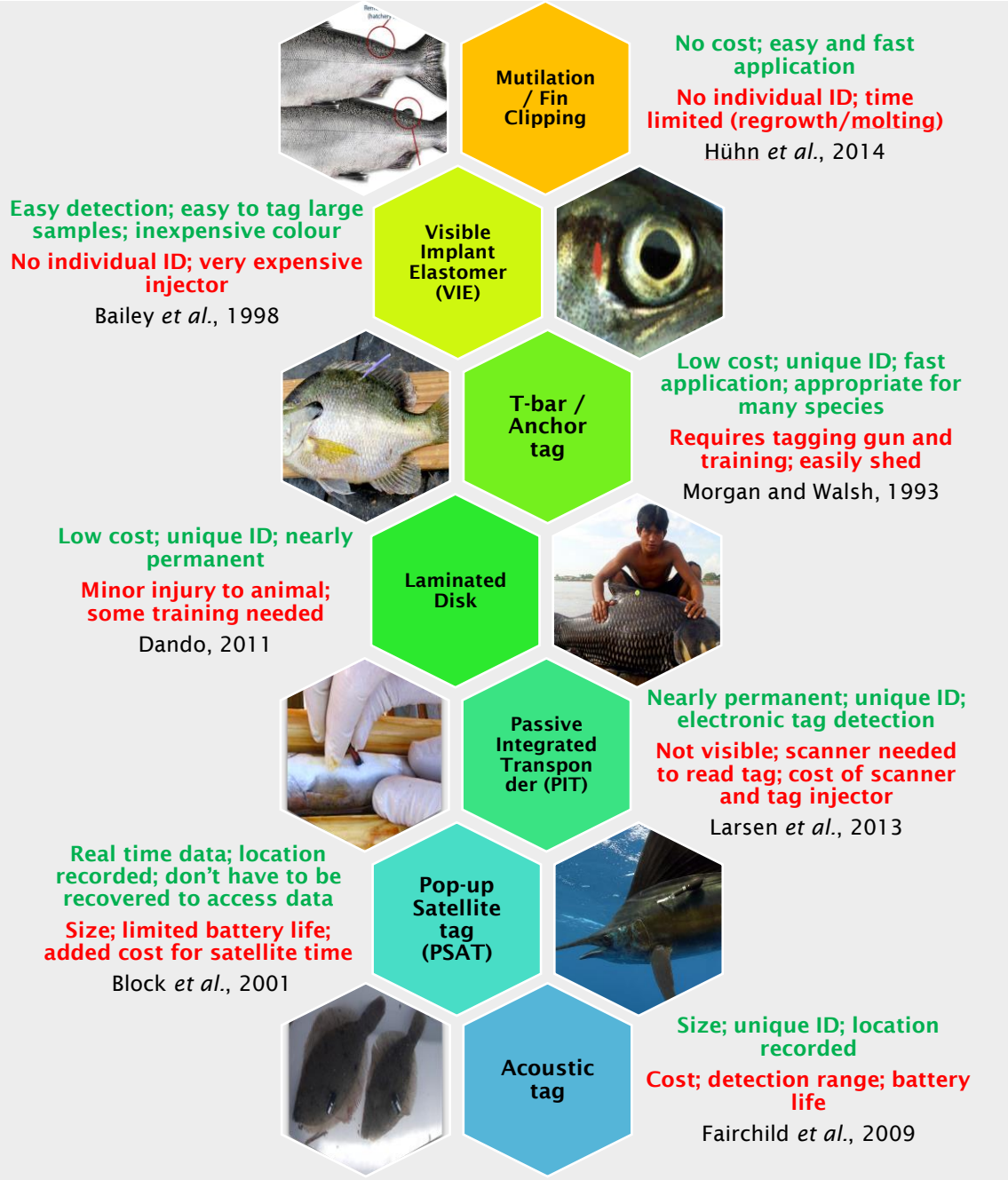


***Figure 1.1*** *– Tag types and some advantages (in green) and disadvantages (in red) of their use in tagging studies.*

Besides the use of artificial tags – mutilation, dyes and physical tags – natural tags/marks have also been used to study fish. Natural tags include meristic and morphometric characters and hard parts of fish body as scales and otoliths. Patterns in fish scales have been used since the 1900s, preferably to study fish because meristic and morphometric marks suffer from the influence of the environment and genetics (McFarlane *et al.*, 1990). Otoliths, i.e. calcium carbonate concretions located in the fish' inner hear, are also a "natural" mark as they register

fish's life history, being used to determine fish's age, delineate stocks, recreate environmental history and deduce migration patterns (Campana, 2005). Technological advances have also helped grow the research possibilities with these natural marks. For example, fish migration patterns can be determined through microchemistry analysis of these marks. The ratio of trace chemical elements, such as Sr:Ca (strontium:calcium), present in otoliths, can be analysed and according to their concentrations it's possible to determine where fish lived (along salinity gradients) during different life stages (Campana, 2005).

## 1.2 | Telemetry studies

In recent years, the most significant advances in tagging and tagging methodologies have come about with the development of electronic tags (Trefethen, 1956; Johnson, 1960; Bridger & Booth, 2003). Biotelemetry is defined as "the remote detection and measurement of a human or animal function, activity, or condition (such as heart rate or body temperature)" (Merriam-Webster, 2017), using transmitters and receivers to send information from the marked individual to the researcher (figure 1.2).

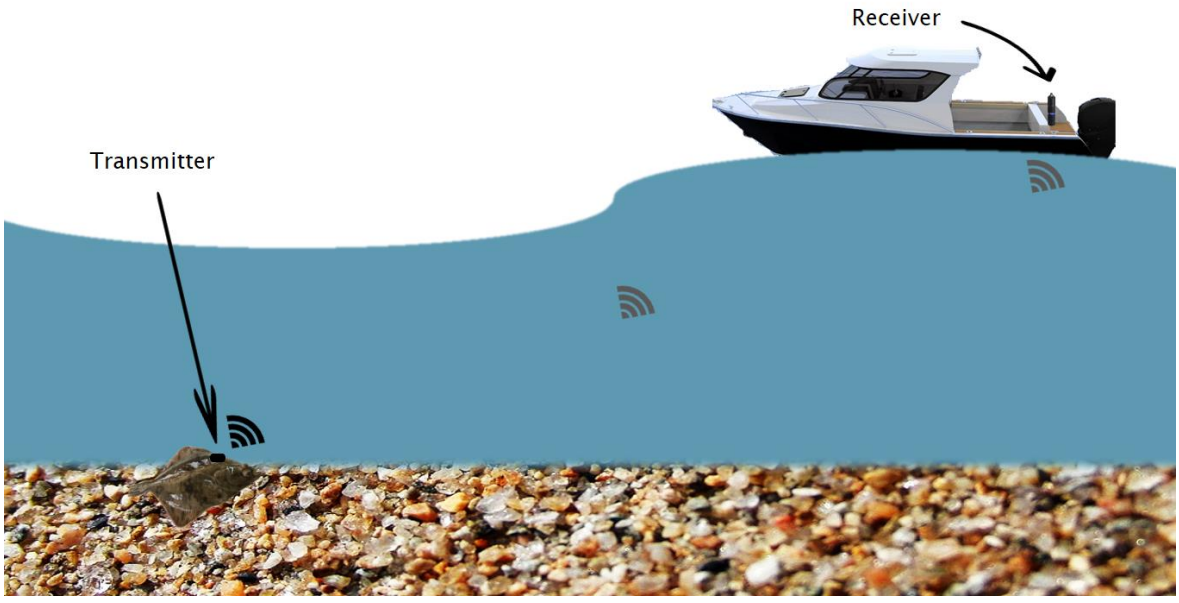


***Figure 1.2** – Externally marked flounder (P. flesus) transmitting information to a receiver in the boat.*

Transmitters, i.e. tags, which are either internally or externally placed on the animals, emit signals through acoustic or ultrasonic waves, at low frequencies (30-300/400 KHz). Tags can either transmit continuously or in coded bursts.

Continuous transmitters emit sequential pings at constant or varying intervals usually correlated to a physical variable, and each tag emits pings at different frequencies allowing to track and monitor different individuals and gather information on each one. Coded transmitters emit a series of pings (burst), which contain a digital identification and (oftentimes) physical data, over a few seconds followed by a delay before emitting the next burst. The choice of delay depends on several factors such as the number of marked animals, swimming speed, and detection range. Coded transmitters are typically used in large scale population studies (Webber, 2009). The relatively low frequencies at which the signals travel minimize absorption and attenuation of the signal while it travels underwater, making it the best method to track and monitor animals in aquatic environments. Besides tracking the marked animals, these tags can be supplied with sensors to measure either environmental conditions (depth, salinity, temperature) and/or physiological variables (heart rate and body temperature) (Cooke, 2008) gathering more data in addition to spatial position.

Acoustic telemetry devices have been used in fishes, crustaceans, cephalopods and mammals, in marine and freshwater habitats (Donaldson *et al.*, 2014; Hussey *et al.*, 2015). These have allowed for an increase in our knowledge on the biology and ecology of aquatic organisms that move freely in conditions inhospitable to human observers (Smircich and Kelly, 2014).

Choosing the appropriate tag, as previously mentioned, is an important step in telemetry studies, being strongly influenced by the target study animal and the objective and/or duration of the study. Since the beginning of its use, this discipline as evolved immensely. The many technological advances have allowed the miniaturization of these marks making it possible to track smaller fish species or earlier life stages of animals previously only tracked in adulthood (Smircich and Kelly, 2014). Although the use of smaller tags has expanded the suite of organisms that can be tracked, it has also raised other issues such as duration of battery life and memory, which can influence the duration of the studies (Pursche *et al.*, 2014). Another aspect to consider before selecting the tag is where and how it will be placed, internally or externally. Both placements have advantages and disadvantages, that vary greatly among species, size and body shapes. Laterally compressed fishes are usually externally tagged or tagged with smaller tags because their peritoneal cavities have less available space to accommodate tags (Moser *et al.*, 2005). To solve this issue, the use of compressed tags has been suggested, instead of the typically cylindrical tags (Mulcahy, 2003). Bigger and

round/fusiform fish usually have the tag implanted in their peritoneal cavity, closer to their centre of gravity, because the internal placement helps with buoyancy and equilibrium, and in addition it avoids effects of drag forces, fouling or entanglement of the tag (Bridger & Booth, 2003).

A key assumption in most tagging studies, including biotelemetry ones is that the tagged animals must be a representative sample of the general population. This means that the tagged fish cannot deviate from the wild population in either behaviour or physiology (Smircich and Kelly, 2014). It is thus important to make sure that both the tag itself and the selected tagging procedure do not affect the natural behaviour nor the physiological condition of the fish.

Tag burden (i.e. the ratio of transmitter weight to fish weight) is one of the greatest concerns in this field of research. Most researchers adhere to a guideline where the tag weight should not be greater than 2% of fish body mass in air (Winter *et al.*, 1983). Although some more recent studies have challenged the "2% rule" showing that this value may be too strict and conservative (Smircich and Kelly, 2014; Larsen *et al.*, 2013), tag burden is still one of the most concerning issues when assessing the movements of small juvenile fish. Tagged fish carry an additional weight that can force them to expend more energy when actively swimming or to make corrections to recover equilibrium and/or buoyancy. These increases in energy requirements can lead to the diversion of energy from somatic or gonadal growth and even from swimming performance (Bégout Anras *et al.*, 2003). The consequence is that fish growth or development might be impaired, which is critical in reproductive migration studies. If tagged fish are not developing as their wild untagged counterparts, it might mean that the data provided by the tagged individuals may not accurately represent wild fish movements or behaviours.

Another concern is the effect of tagging on social interactions because they partially determine fish movements and habitat use. All procedures associated with tagging a fish (capture, handling, tagging and recovery) can cause stress which can impact and change many social behaviours: aggression, position in dominance hierarchies, competition, parental care and shoaling, amongst others (Jepsen *et al.*, 2015). It can likewise affect feeding behaviour, either due to loss of social status or due to direct interference of the tag e.g. gastrically implanted transmitters, impacting fish's growth, development, condition and survival. Predation risk is also a concern because in some instances tagging can compromise fish's swimming ability, diminish their evading capabilities, and increase the prey's visibility, especially when fish are externally marked (Pursche *et al.*, 2014).

For a long time, survival was the most common criteria to determine the success of a tagging experiment. However, cause of death is not always related to tagging because fish can die from causes not related to the tagging procedures. Moreover, even if mortality can be used as a reliable indicator of an adverse effect, it should not be assumed that surviving fish were not affected by the procedures. If the goal is to gather unbiased data, the loss of data from an individual that did not die is preferable to the gathering of data from animals suffering sub-lethal effects (compromised swimming capabilities, growth and development, infection) that alter fish behaviour and physiology (Mulcahy, 2003).

Another critical factor for these studies is tag retention. The loss of a tag is not only a loss of possible data but can also be misinterpreted as mortality and bias the results of the study. It cannot be assumed that a high retention rate of a tag in a fish species means that such tag or attachment methodology will have the same results, in respect to retention rate, for all fish species (Broadhurst *et al.*, 2009; Jepsen *et al.*, 2015), or that the overall effects on fish's behaviour and physiology are the same across species.

Prior to any field-based telemetry study it is thus important to test the tagging techniques and procedures in the target species, and to investigate and assess the potential effects on fish behaviour, physiology and survival. The methods of capture, confinement, transportation and handling of the fish prior to and during tagging are also of importance as they constitute additional sources of stress. In this respect, careful procedures should thus be taken to minimize fish stress and increase the overall success of the tagging study.

### 1.3 | Fish welfare in telemetry studies

Fish welfare is a growing concern in fish culture and research, as well as ethical investigation (Popovic *et al.,* 2012). Nowadays, the use of anaesthetic agents is already considered common practice in aquaculture and research, as it makes routine procedures less stressful for fish (Olfert *et al.*, 1993; AFS, 2004; Carter *et al.*, 2011; Popovic *et al.,* 2012).

Pain perception by fish remains a controversial subject (Carter *et al.*, 2011; Sneddon, 2012; for counterarguments, see: Rose, 2002; Sneddon et al., 2003; and Braithwaite & Boulcott, 2007), but there is enough evidence that anaesthetics and analgesics help to improve the fish wellbeing (Carter *et al.*, 2011; Sneddon, 2012), which led to an increase in the number of studies evaluating their use on fish, especially of anaesthesia procedures. Even so it is still not possible to establish a

general anaesthesia protocol because the anaesthetic dose to use varies with species, fish condition, required degree of anaesthesia and type of anaesthetic, among other factors. The use of analgesics is even more recent, and hence there are still no valid analgesic protocols. Morphine and lidocaine have been recommended but so far only in a study involving the rainbow trout (see review by Sneddon, 2012).

The lack of knowledge in this field makes it necessary, when a study requires invasive procedures, to investigate the most efficient dosages of anaesthetic and analgesic to use on the study species.

### 1.4 | Model species

Many marine fish species have complex life histories in which transitions between life stages coincide with shifts in habitat use. One typical pattern includes spawning offshore, colonization of coastal or estuarine nursery areas by late larvae/juveniles and recruitment back to the coast as adults (Beck *et al.*, 2001; Able, 2005).

This is the case of the European flounder (*Platichthys flesus* Linnaeus, 1758), a coastal species which is an important component of the demersal fish assemblages in European waters, from Norway to Morocco (Nielsen, 1986; Freitas *et al.*, 2009; Morais *et al.*, 2011). With rises in seawater temperatures, associated with climate change, the southern geographical limit of this species seems to have contracted and it is presently only found in the central and northern coastal areas of Portugal (Cabral *et al.*, 2007; Freitas *et al.*, 2009).

Through its northern distribution, this flatfish species has a higher recreational value, being deemed a popular sport fish. In the Baltic and Danish waters, flounder is a target fisheries species and some management measures have been implemented to protect the stocks (Skerrit, 2010). Similar to most European countries, in Portugal there is no active commercial flounder fishery and the species is usually caught as by-catch by commercial demersal fishers or targeted by artisanal fisheries (ICES, 2010).

This species has been found in freshwater, estuarine and coastal ecosystems, and adjacent seas (Nielsen, 1986; Skerrit, 2010; Morais *et al.*, 2011). It is a marine migrant species (Elliott *et al.*, 2007), spawning in marine waters during the winter and early spring months, then moving to shallower waters, such as estuaries, that they use as feeding and nursery grounds (Summers, 1979; Freitas *et al.*, 2009; Morais *et al.*, 2011). Their eggs hatch in the sea (Grioche *et al.*, 1997) and, larvae and young-of-the-year use selective tidal transport to migrate into the estuaries

(Morais *et al.*, 2011; Le Pichon *et al.*, 2014). According to most distribution and abundance studies, newly settled flounder prefer low salinity areas as nurseries (Cabral *et al.*, 2007; Ramos *et al.*, 2010), that can range from brackish waters to freshwater river habitats (Kerstan, 1991; Jager, 1999), until the end of autumn when they seem to migrate to more coastal areas (Morais *et al.*, 2011). Older fish are, usually, more widely distributed throughout the estuaries (Kerstan, 1991; Jager, 1998; Cabral *et al.*, 2007). Although this is the most accepted description of *P. flesus* life cycle, this species may also present alternative life history patterns: in the Minho estuary and some estuaries in France, Daverat *et al.* (2011, 2012) and Morais *et al.* (2011) found, based on otolith microchemistry analysis, that some flounder can spawn in estuarine waters. This led them to hypothesize that the flounder catadromous life cycle might be facultative, at least in the above-mentioned estuaries. In the Baltic Sea, this species has evolved in such a way that there are two sympatric flounder populations, where one produces pelagic eggs while the other produces demersal eggs that allow for a successful reproductive effort according to the salinity conditions they inhabit (Nissling *et al.*, 2002; Florin and Höglund, 2008). Moreover, Johnston (1981); Beaumont and Mann (1984); and Le Pichon *et al.* (2014) saw flounder as old as 3 and 5 years, respectively, that had always remained in freshwater habitats and some juvenile and adult flounder have even been caught 70 km from the river mouth (Morais *et al.*, 2011), when it's thought that at the end of the summer the young-of-the-year start migrating downstream.

The estuaries along the Portuguese coast have been identified as important nursery grounds for flounder, particularly Ria de Aveiro lagoon, and the Mondego, Douro, Lima and Minho estuaries (Cabral *et al.*, 2007; Vasconcelos *et al.*, 2008; Freitas *et al.*, 2009; Ramos *et al.*, 2010). Higher flounder densities are reached in the spring/summer with the arrival of young-of-the-year, as it also happens in other European estuaries (Freitas *et al.*, 2009). In the Douro estuary, however, the highest annual densities were recorded in autumn (Vinagre *et al.*, 2005). Vinagre *et al.* (2005, 2008) studied the population of *P. flesus* in the Douro estuary, from the coastal area to the upper estuary limited by the Crestuma-Lever dam (21.8 km upstream from the river mouth), and while the seasonal abundance patterns were similar to the expected, with an increase in abundances in the summer and a decrease in the winter, they were more abundant in mesohaline waters, rather than oligohaline or fresh waters.

In this context, acoustic tagging can be a useful tool to namely study adult flounder

movements during the reproductive season, clarifying spawning migration pathways between the coast and the estuary. It can also be a powerful tool to collect clues on juvenile movements – their preferred habitat within the estuary (fresh or brackish waters) and fine-scale movements. However, tagging studies with *P. flesus* are scarce, with Dando (2011) being one of the few studies disclosing this species' site fidelity, homing and spawning migration patterns in SW England through mark and recapture experiments. In fact, to the best of our knowledge there are only two studies that report the use of electronic tags on *P. flesus*: Wirjoatmodjo & Pitcher (1984) studied the movements, interpreted as feeding behaviour, at each tidal state of mature flounder (3 years old, >160g) and Le Pichon *et al.* (2014), studied summer movements of late juveniles. An acoustic telemetry study is one of the ways to better understand flounder life cycles. By tracking juvenile and adult fish we can learn more about their use of different habitats along the sea/costal-estuarine-river areas throughout their life and about their reproductive migration patterns.

## 1.5 | Aims and outline of this thesis

This thesis' main goal was to determine the acoustic tagging method that least affects flounder and with the highest retention rate, allowing the collection of data for as long as possible. To accomplish this, a suite of other specific objectives were addressed:

i. operationalize all aspects of fish maintenance and manipulation associated with fish tagging in laboratory, including anaesthesia and analgesia;
ii. assess the effects of the tagging procedures on fish physiology and behaviour;
iii. evaluate tag loss and its causes;
iv. select the procedure, under a laboratory setting, with the least negative effects on fish welfare and highest tag retention rate.

Chapter 2 describes the results of a pilot study testing the procedures related with Anaesthesia and Analgesia. The results of this assay were then used in the tagging experiments.

Chapter 3 describes the optimized procedures and the full protocol for conducting the tagging as well as the evaluation of short-term effects in flounder behaviour, physiological condition and survival.

Chapter 4 focuses on the pathology of captive flounder; namely on the development of skin ulcers. Disease prevalence is examined in relation to sex, life stage, stock and experimental treatment.
Finally, chapter 5 provides a general discussion with guidelines and recommendations for implementing effective tagging of flounder *P. flesus* in the field.

## 1.6 | Ethical statement

All experiments conducted in this study were carried out at Biotério de Organismos Aquáticos (BOGA, CIIMAR) aquatic animal facilities and have been approved by the CIIMAR ethical committee and by CIIMAR Managing Animal Welfare Body (ORBEA) according to the European Union Directive 2010/63/EU "on the protection of animals used for scientific purposes".

# Chapter 2 | Determining MS-222 efficient dosage to tag *Platichthys flesus* and lidocaine's effectiveness as a painkiller

## 2.1 | Introduction

Anaesthetic agents are often used in aquaculture and research with aquatic animals because even routine tasks (e.g. capture, handling, etc) can induce physiological stress responses. The use of anaesthetics diminishes the acuteness of said stress response, helps to prevent any damage fish might cause itself, allows the safe use of a greater number of specimens and the performance of longer procedures (Ackerman *et al.*, 2005; Sneddon, 2012). Besides, even though the fishes' ability to centrally process pain is often contested, with the growing concerns towards animal welfare it is recommended that, in procedures assumed to induce pain, the painful stimulus should be mitigated by administrating anaesthetics and analgesics (Sneddon, 2012). Good animal welfare requires, amongst other necessities, the humane handling and killing of fish (OIE Resolution, Article 7.1.1.).

Different types of procedures demand different stages of anaesthesia (Sneddon, 2012). Routine tasks, rapid and non-invasive procedures, can be performed under light anaesthesia; however longer and more invasive procedures require deeper levels of anaesthesia, namely surgical or deep anaesthesia (Sneddon, 2012). Deeper anaesthetic stages, characterized by a decreased respiration rate (weak opercular movements) and a total loss of equilibrium and of reactivity (Popovic *et al.,* 2012), should not be maintained for longer than 10 min and artificial gill ventilation is an advised precaution. Artificial gill ventilation will not only help prevent hypoxia, by increasing passive gas exchange, but can also help maintain the anaesthetic stage if, instead of using 'clean water', the water irrigating the gills is laced with a low dose of anaesthetic (Summerfelt & Smith, 1990; Ross & Ross, 2008; Carter *et al.*, 2011).

An anaesthetic can be a physical or a chemical agent; it starts by inducing a calming effect and, depending on concentration and time of exposure, it can cause successive loss of equilibrium, mobility, consciousness, and reflex action (Summerfelt & Smith, 1990; Keene *et al.*, 1998). In fish studies, an anaesthetic should be water soluble, have a rapid induction and recovery time, and provide adequate immobilisation and analgesia for the duration of the procedures. However, anaesthetics' efficacy hinges on several environmental (water quality, pH, oxygenation, temperature, and salinity) and biological (age, developmental stage, sex and reproductive condition, size, body condition and weight, lipid content,

growth and physiological status, health and fish species) factors (Ackerman *et al.*, 2005; Weber *et al.*, 2009; Sneddon, 2012). Therefore, it is extremely important to carefully monitor the fish as they go through the different stages of anaesthesia. By watching, it is possible to learn the changes the anaesthetic causes on the fish demeanour, activity, equilibrium, eye movement, gill ventilation and heart rate, reactivity and muscle tone; which will then help to assess if the fish has reached the desired anaesthetic stage (Carter *et al.*, 2011; Sneddon, 2012). Weber *et al.*, (2009), after performing a pilot study, reported that flatfish go through the same changes as other fish during induction and recovery. However, assessing swimming movements and equilibrium maintenance in these fishes is not as easy as when working with round-fish and so the care given to observations during anaesthesia induction grows in importance. Hence, some authors choosing to turn flatfish's uneyed side upwards to verify if they have indeed lost their ability to regain equilibrium (Malmstrøm *et al.*, 1993).

A few anaesthetics are commonly used in fish, and experimental studies have been performed to explore induction, recovery, pharmacokinetics and side effects. Tricaine methane-sulphonate (MS-222) is one of them, and one of the most used and recommended in research and fish culture, especially for routine operations and for more invasive procedures (Malmstrøm *et al.*, 1993; Sneddon, 2012; Popovic *et al.,* 2012). MS-222 is usually administered via immersion, enters the body through the gills, and anaesthetizes by blocking neuronal signal transmission peripherally to the central nervous system. It induces a rapid and deep stupor with short recovery times (McFarland, 1960; Mattson & Riple, 1989; Malmstrøm *et al.*, 1993; Ackerman *et al.*, 2005), and has few side effects. The dosages vary greatly among species and desired anaesthetic stage (table 2.1); the concentrations used are usually between 50 and 400 mg/L (Sneddon, 2012), with anaesthetics doses ranging between 25 and 100 mg/L and lethal dosages between 400-500 mg/L. Even doses as low as 50 mg/L must be used cautiously because long exposures can be deadly. The higher doses are used to euthanize the specimens with the least possible anxiety, pain and distress (Sneddon, 2012).

***Table 2.1 – MS-222 dose estimates (mg/L); induction and recovery times in minutes for various fish (test fish) and applicability for the test fish. Adapted from Ackerman et al., 2005 and Popovic et al., 2012. N/S – Not stated.***

| Dose (mg/L) | Induction time (min) | Recovery time (min) | Test fish | Indication | References |
|---|---|---|---|---|---|
| 25 – 100 | < 3 | < 10 | Salmonids, Carp, Minnows | N/S | Bell & Blackburn, 1984; Gilderhus & Marking, 1987; McFarland & Klontz, 1969; Schoettger & Julin, 1967; Sylvester & Holland, 1982; Yesaki, 1988 |
| 80 – 100 | 2.6 – 6.8 | 2.5 – 1.2 | Tilapia | N/S | Ferreira *et al.*, 1979; Ross & Ross, 1984 |
| 100 – 230 | 22 – 30 | Immediate | American eel | Stage II | Prieto *et al.*, 1976; Hinton & Loyacano, 1978; Ross & Ross, 2008 |
| 66 | 4-6 | N/S | American paddlefish | Deep stage, blood withdrawal | Cittinger *et al.*, 1992; Ross & Ross, 2008 |
| 60 – 75 | 0.9 – 2.7 | 3.7 – 7.2 | Atlantic cod | Deep stage | Mattson & Riple, 1989; Ackerman *et al.,* 2005; Ross & Ross, 2008; Zahl *et al.*, 2009 |
| 250 – 480 | < 5 | < 10 | Atlantic halibut | Deep stage | Malmstrøm *et al.*, 1993; Ackerman *et al.*, 2005; Ross & Ross, 2008 |
| 70 | 6 | 10 | Black sea bass | Stage II & III, blood withdrawal, biopsy, tagging | King *et al.*, 2005; Ross & Ross, 2008 |
| 70 – 100 | 1.9 – 4.7 | 1.2 – 1.7 | Blackspot seabream | Stage III | Maricchiolo & Genovese, 2011 |
| 100 | 7 – 12 | N/S | Brook trout | Stage II | Houston *et al.*, 1971; Ross & Ross, 2008 |
| 250 – 350 | N/S | N/S | Carp fry | Deep stage | Jain, 1987; Ross & Ross, 2008 |
| 90 – 250 | 3 – 4.5 | 1.7 – 5.3 | Channel catfish | Deep stage | Coyle *et al.*, 2004; Small & Chatakondi, 2005; Welker *et al.*, 2007 |
| 25 – 100 | 3.7 – 4.9 | N/S | Common carp | Sedation, transport, deep stage | Houston *et al.*, 1973; Berka, 1986; Takeda *et al.*, 1987; Dziaman *et al.*, 2005; Ross & Ross, 2008 |
| 50 – 100 | 1.3 – 4.3 | N/S | European perch | Sorting & blood withdrawal | Jacquemond, 2004; Velíšek *et al.*, 2009 |
| 75 – 100 | 3 | 2.4 – 6.5 | Fathead minnow | Stage III, blood withdrawal | Palić *et al.*, 2006 |
| 25 – 50 | 1 | > 60 | Gilthead seabream | Transport, deep stage | Cubero & Molinero, 1997; Ortuno *et al.*, 2002 |
| 20 – 75 | N/S | N/S | Grass carp | Sedation, transport, deep stage | Schramm & Black, 1984; Berka, 1986; Ross & Ross, 2008 |
| 70 – 100 | 2.6 – 5.7 | 2.4 – 2.6 | Greater amberjack | Stage III | Maricchiolo & Genovese, 2011 |
| 20 – 120 | N/S | N/S | Mullet | Sedation, transport, deep stage | Dick, 1975; Sylvester, 1975; Ross & Ross, 2008 |

| Dose (mg/L) | Induction time (min) | Recovery time (min) | Test fish | Indication | References |
|---|---|---|---|---|---|
| 60 – 150 | 1.7 – 3.3 | 50.2 – 6.2 | Rainbow trout | Light to deep stage, blood withdrawal, laboratory studies | Wedemeyer, 1969, 1970; Soivio *et al.*, 1977; Wagner *et al.*, 2002, 2003; Pirhonen & Schreck, 2003; Coyle *et al.*, 2004; Holloway *et al.*, 2004; Ross & Ross, 2008 |
| 55 | 3 | 10 | Red drum | Stage IV | Massee *et al.*, 1995; Ross & Ross, 2008 |
| 50 – 100 | N/S | N/S | Red seabream | Deep stage | Ishioka, 1984; Ross & Ross, 2008 |
| 30 – 150 | ≤ 1.6 | ≤ 2.1 | Sailfin silver molly | Stage I, loss of equilibrium | Küçük, 2010 |
| 80 – 135 | 4 – 12 | 3 – 19 | Salmonidae | Deep stage, blood withdrawal | Strange & Schreck, 1978; Hill & Forster, 2004; Ross & Ross, 2008 |
| 70 | N/S | N/S | Sea bass | Sorting | Chatain & Corrao, 1992 |
| 50 – 100 | 2.3 – 7.5 | 2.8 – 3 | Senegalese sole | Stage III | Weber *et al.*, 2009 |
| 60 – 100 | N/S | N/S | Silver seabream | Blood withdrawal, stage III | Ryan, 1992; Ross & Ross, 2008 |
| 110 – 150 | < 3 | < 10 | Striped bass | Deep stage | Henderson-Arzapalo *et al.*, 1992; Lemm, 1993; Ackerman *et al.*, 2005; Ross & Ross, 2008 |
| 80 – 250 | 4 – 15 | 3 – 7 | Sturgeon species | Blood withdrawal, surgical examination & biopsy of gonads | Conte, 1988; Hernandez-Divers *et al.*, 2004; Divers *et al.*, 2009; Di Marco *et al.*, 2011; Feng *et al.*, 2011; Matsche, 2011; Matsche *et al.*, 2011 |
| 40 – 150 | 3 | N/S | Tilapia | Sedation, deep stage, blood withdrawal | Balfry *et al.*, 1997; Smith *et al.*, 1999; Coyle *et al.*, 2004; Barreto *et al.*, 2007 |
| 25 – 200 | N/S | N/S | Tench | Deep stage | Randall, 1962 |
| 40 | > 1.5 | N/S | Brook trout | Tagging | Smircich & Kelly, 2014 |
| 500 | 2 | N/S | Winter flounder | Tagging | Fairchild *et al.*, 2009 |
| 25<br>80 | 15<br>5 | N/S | English sole | Sedation<br>Surgery, tagging | Moser *et al.*, 2005 |
| 130 | 2.5 – 3.2 | 3.2 – 11.6 | Pacific halibut | Deep stage, tagging | Loher & Rensmeyer, 2011 |

Analgesics are used to reduce pain, by blocking nociceptive transmission, and can either be applied topically or be injected before the painful stimulus. They promote wellbeing and therefore advance recovery. Even though their use is common in clinical and veterinary sciences there are only a few references to their use in fish studies (see review by Sneddon, 2012). Moreover, of the analgesics already tested in fish not all were considered safe to use *in vivo*. The use of lidocaine in rainbow trout, however, has been deemed safe, with 1mg/kg being the most effective dose to reduce physiological and behavioural responses to pain (Sneddon, 2012). Its effect lasts 30 to 60 minutes and its dosage should not exceed 2 mg/kg.

However, studies on anaesthetics and analgesics were done on very few species and the use of these on other, non-validated species, should be done with care and caution. Anaesthetic efficacy should be tested using a small number of fish, starting with low dosages and increasing them until achieving the effective dosage (Sneddon, 2012).

Many authors suggest that an ideal anaesthetic should induce deep stages of anaesthesia in under 3 min and allow for recovery times smaller than 5 min (Marking & Meyer, 1985; Iwama & Ackerman, 1994; Carter *et al.*, 2011). For invasive procedures, like through-body and/or intracoelomic tagging, fish need to be sedated to a surgical stage (Sneddon, 2012). Therefore, the effective dosage of anaesthetics was determined to follow these recommended times. This assay had two goals: (1) to define the lowest and most efficient concentration of MS-222 to anaesthetise flounder *Platichthys flesus* to the desired anaesthesia stage (i.e. surgical anaesthesia (*sensu* Sneddon, 2012), and (2) to assess the efficacy of an analgesic (lidocaine) dosage of 1mg/kg for this species.

## 2.2 | Materials and methods

### 2.2.1 | Experimental animals

Flounder (*P. flesus*) were originally captured in the lower Douro estuary in April 2017 (see Chapter 3 for further details) and transported to BOGA where they were kept in a quarantine. After a period of 25 days, 9 fish were randomly selected for an anaesthesia and analgesia assay. Prior to the beginning of the assay, fish were starved for 4 days.

The flounder used in this experiment had a mean body weight of 194.56 g ± 61.40 and mean total length 26.60 cm ± 2.89.

### 2.2.2 | Anaesthetic and analgesic

The anaesthetic agent used was MS-222 (Tricaine Methane Sulphonate, Pharmac), and the concentrations tested are shown in table 2.2. Initial anaesthetics concentrations used in this assay were based on concentrations used in similar tagging procedures in other flatfish species. Fairchild *et al.* (2009) anaesthetised juvenile (<19cm) winter flounder (*Pseudopleuronectes americanus*) with 0.5 g/L of MS-222 for 2 min and Moser *et al.* (2005) anaesthetised English sole (*Pleuronectes vetulus*), larger than 27 cm, also with MS-222 in two different baths, first a sedative dose of 0.025 g/L for 15 min followed by a bath of 0.08 g/L for 5 min. The maintenance bath concentration was chosen in order to keep the fish sedated but without deepening the anaesthesia stage. Thus, the use of a dosage of approximately 50% of the initial induction concentration tested. The analgesic used was lidocaine (Lidocaine 2%, B. Braun) at a concentration of 1mg/kg.

MS-222 and buffer were dissolved in 10L (induction bath, initial concentration was 0.070 g/L) and 8L (maintenance bath, 0.038 g/L) of strongly aerated saltwater from the quarantine system (35.4 psu, 19.9˚C), a few minutes before starting the experiment.

***Table 2.2 – Anaesthetic concentration in the induction and maintenance bath where fish were anaesthetised. (-) – the fish was not placed in the bath; (*) – marks the reinforcement of the maintenance bath with 0.2 g of MS-222 and 0.2 of buffer before anaesthetising fish 7 through 9.***

| Fish ID | MS-222 (g/L) | |
|---|---|---|
| | Induction Bath | Maintenance Bath |
| 1 | 0.070 | - |
| 2 | 0.090 | 0.038 |
| 3 | 0.110 | 0.038 |
| 4 | 0.120 | 0.038 |
| 5 | 0.120 | 0.038 |
| 6 | 0.120 | 0.038 |
| 7 | 0.120 | 0.038(*) |
| 8 | 0.120 | 0.038 |
| 9 | 0.120 | 0.038 |

[1] The MS-222 concentration of the induction and maintenance baths are the initial values, before a fish was placed into the bath. Concentrations are mostly overestimated because each fish partly assimilates the anaesthetic present in the bath.

2.2.3 | Experimental design

The loss of equilibrium is one of the most obvious signs that fish are going from lightly sedated to deeper stages of anaesthesia, however, in flatfishes this is not easy to observe naturally. Therefore, with the help of a fish net, when it seemed that the flounder were reaching those stages they were carefully turned uneyed-side up to see if they still had the ability to regain their normal posture or if they no longer tried to right themselves (figure 2.1).

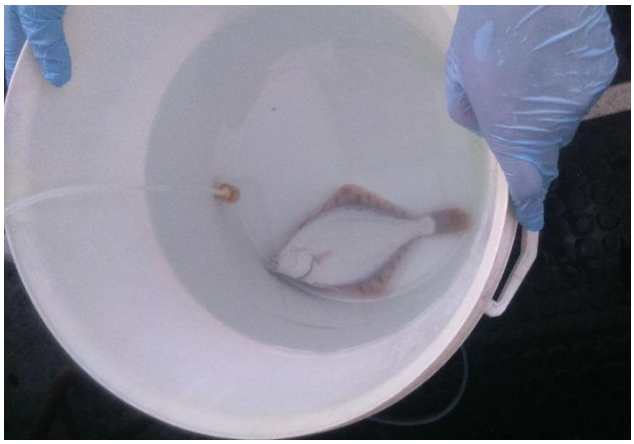

***Figure 2.1*** *– Flounder, in induction bath, turned upside-down to assess loss of equilibrium.*

One by one, flounder were transferred from the quarantine tank to the induction bath, where fish were held individually. As soon as they were placed there, the time required to lose equilibrium and the induction time were recorded to the closest second with a stopwatch. Loss of equilibrium was assessed, for the first time, 90 seconds after fish were placed in the induction bath. After, if loss of equilibrium had not yet occurred it was re-evaluated at, approximately, 15 seconds intervals. The induction time was recorded when the gill ventilation decreased and seemed almost shallow. When the gill movements had decreased flounder were taken from the induction bath and were measured (total length) to the closest mm and weighted to the g. Then, they were placed in a padded surgical cradle where a light anaesthetic dosage, hereafter called maintenance anaesthesia (see table 2.2), was provided to maintain the surgical anaesthesia stage that the flounder had reached (figure 2.2).

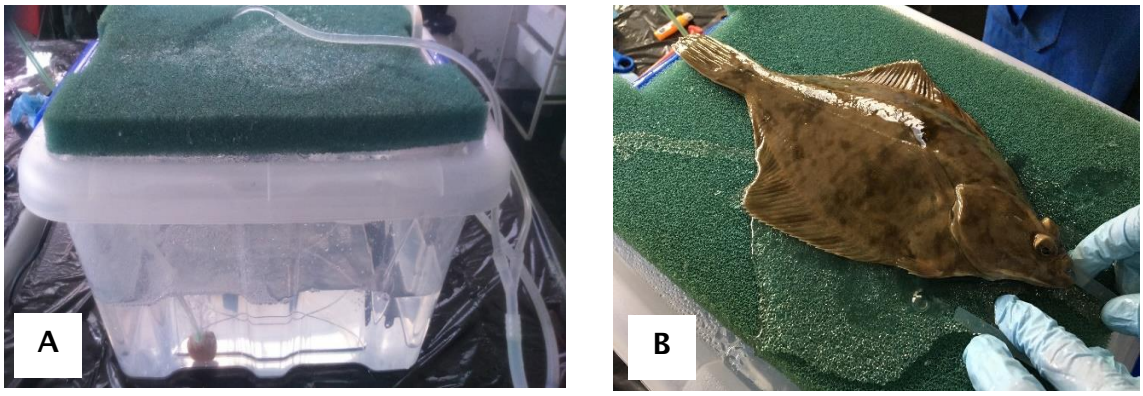


***Figure 2.2** – **(A)** Surgical cradle with maintenance bath; **(B)** Flounder on surgical cradle being dosed with water from the maintenance bath through the mouth.*

According to the flounder weight, the quantity of lidocaine was measured and then injected into the dorsal musculature of the flounder' eyed side (see table 2.3 and figure 2.3). A few seconds after administering the lidocaine, the flounder's pain perception was assessed by pricking, with a needle, the analgised area. Fish were also handled to see if they struggled.

***Figure 2.3** – Administration of lidocaine in the dorsal musculature of the flounder.*

After these two assessments were made the flounder were transferred to a recovery tank (10L of well aerated system water), with their uneyed side upwards, and the times required for equilibrium restoration and for recovery were recorded to the nearest second. Recovery time was defined as the total time since they were placed in the recovery bath until respiration rate increased and started to swim, either naturally or when prompted.

Each flounder underwent the anaesthesia and analgesia testing only once. If an

anaesthetic dose did not cause equilibrium loss after 3 min (the desired full induction time) on the first flounder, the flounder was taken directly to the recovery tank and the anaesthetic concentration was increased (as in fish 1 through 4, table 2.2) and tested on a subsequent individual. At the end of the assay fish were euthanised with an overdose of 2-phenoxyethanol.

## 2.3 | Results and discussion

Anaesthetics efficacy is a subjective variable, depending on the handler, the fish, procedures and environmental conditions, amongst other parameters (Burka *et al.*, 1997; Iversen *et al.*, 2003).

The average time to reach each anaesthesia and recovery stages for each anaesthetic concentration are shown in figure 2.4. The anaesthesia dose of 0.07g/L after 180s did not even cause a loss of equilibrium. Increasing the dosage to 0.09g/L caused equilibrium loss but the induction time (310s), was longer than the optimal defined for this study and the fish did not seem to reach the required depth of anaesthesia. The increase to 0.11g/L, resulted in induction and recovery times closer to the desired but flounder response to the needle pricking and handling was still strong which could possibly hinder tagging (table 2.3). The last dosage, increase to 0.12g/L, resulted in induction and recovery times close to the parameters set, and the response to needle pricking and handling was either non-existent or negligible, see table 2.3. Induction times were greater than the desired 3 min, averaging at 3min and 38s, and recovery times were smaller than 5 min, an average of 4min and 20s. The concentration of the maintenance bath in the surgical cradle was kept constant (0.0375 g/L) until the sixth fish. After finishing the procedure with the sixth fish, the maintenance bath was reinforced with 0.2 g of anaesthetic (and of buffer) due to observations of increased mobility of flounders while in the surgical cradle. The recovery time of the seventh flounder (8min) was far greater than the times registered for the other flounder. The longest recovery time registered with the other flounder (Fish ID 4 to 6) kept in a lower maintenance anaesthesia concentration, was 4min and 30s, meaning that this reinforcement was excessive. Given the effect of the reinforcement of the maintenance bath on flounder recovery it might be best to do new baths, with the initial concentration, or smaller reinforcements, which will also decrease the risk of overdosing.

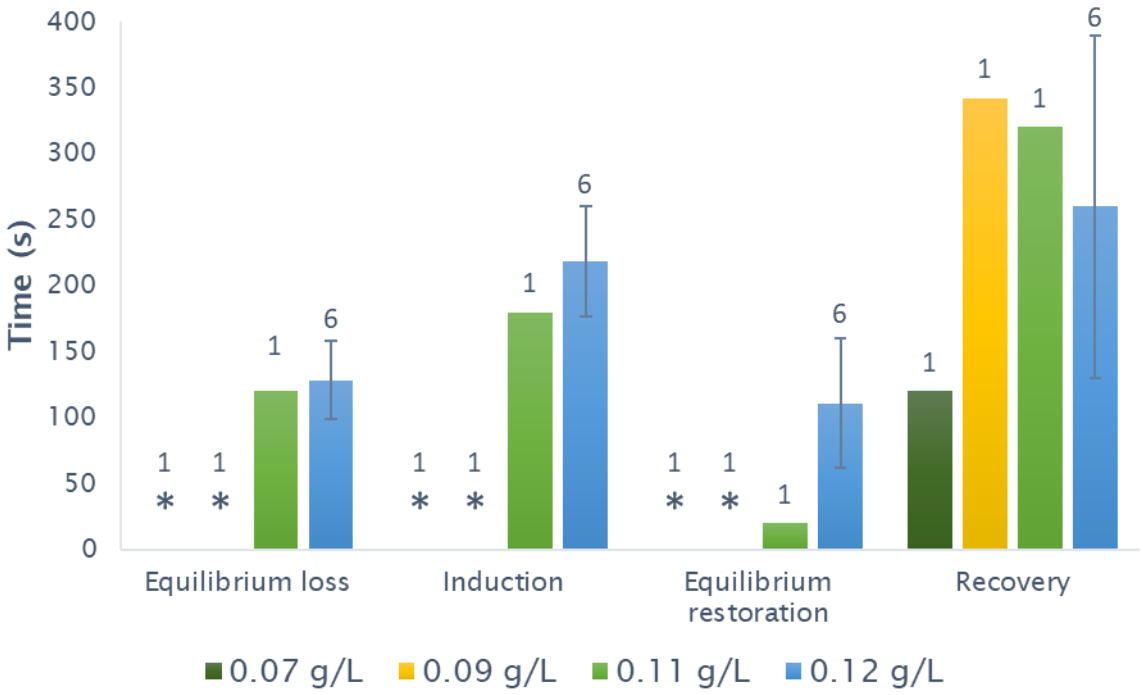


***Figure 2.4** – Average time ± SD (in seconds) to reach each of the studied phases of induction and recovery according to the anaesthesia dosages tested. Sample size (n) is stated above each column. (*) – no effect.*

***Table 2.3 – Anaesthesia, initial induction bath concentration, and analgesic dose (1 mg/kg) administered to each fish and observation of their reactions to handling and to a pain inducing stimulus (needle pricking). (-) – the fish was not placed in the bath.***

| Fish ID | Induction Bath (MS-222 g/L) | Lidocaine (µL) | Reactions to | |
|---|---|---|---|---|
| | | | Handling | Needle Pricking |
| 2 | 0.09 | 12 | Yes | Yes |
| 3 | 0.11 | 8.5 | Yes | Yes, both testing sites |
| 4 | 0.12 | 10 | Light | Yes, site farther from analgised area |
| 5 | 0.12 | 5 | Light | Yes, site farther from analgised area |
| 6 | 0.12 | 6 | Light | No |
| 7 | 0.12 | 12.5 | Light | No |
| 8 | 0.12 | 11 | No | No |
| 9 | 0.12 | 15 | No | No |

The use of analgesics appears to effectively numb the area where they are applied diminishing fishes' response to painful stimuli and keeping them calm, without struggling, during the necessary procedures.

## 2.4 | Conclusion

MS-222 and lidocaine are effective on *P. flesus* under the conditions tested (size range: 26.83±3.53 cm and 200±73.13 g; and environmental conditions: 35.4 psu, 19.9°C) inducing anaesthesia stage III plane, diminishing pain responses and easing fish handling and possibly tagging procedures, at concentrations of 0.12 g/L and 1 mg/kg, respectively, with induction and recovery times close to 3 and 5 min.

# Chapter 3 | Short-term effects of tagging in behaviour, physiology and survival of the European flounder *Platichthys flesus* (L.)

## 3.1 | Introduction

The European flounder *Platichthys flesus*, is a marine flatfish species with a complex life history, involving spawning and hatching at sea, a pelagic larval phase marked by metamorphosis and colonization of fresh or brackish habitats during the larval/juvenile stage (Ramos *et al.*, 2010; 2017). Although much has been known on this species' ecology, recent studies have hypothesized that *P. flesus* might also spawn inside estuaries (Morais *et al.*, 2011; Daverat *et al.*, 2011, 2012). Like most European countries, Portugal lacks an active commercial flounder fishery and the species is usually caught as by-catch by commercial demersal fishers or targeted by artisanal fisheries (ICES, 2010).

Scientific knowledge of fish migrations and spatio-temporal distributions of fish populations has greatly advanced with the use of electronic tags for a variety of species (e.g. DeCelles & Cadrin, 2010; Le Pichon *et al.*, 2014; Dance & Rooker, 2015; Furey *et al.*, 2013; Fairchild *et al.*, 2013). Most telemetry studies with flatfish have used external mounting procedures to study adult spawning movements and late juvenile seasonal distribution patterns, habitat use and residence times (DeCelles & Cadrin, 2010; Fairchild *et al.*, 2013; Furey *et al.*, 2013; Le Pichon *et al.*, 2014; Dance & Rooker, 2015). More recently, technological advances have allowed the tracking of increasingly smaller individuals (11 cm in Fairchild *et al.*, 2009; Le Pichon *et al.*, 2014). However, tagging effects remain poorly studied for a number of species and tagging methods.

To the best of our knowledge, a careful evaluation of the effects of tagging in flatfishes has only been done for *Pleuronectes vetulus* (Moser *et al.*, 2005), *Solea solea* (Bégout Anras *et al.*, 2003), *Paralichthys dentatus* (Fabrizio & Pessutti, 2007) and *Hippoglossus stenolepis* (Loher & Rensmeyer, 2011). The study by Bégout Anras *et al.*, (2003) is the only case where the effects of external tagging are analysed, namely on sole (*S. solea*) growth. The other 3 studies evaluated the effects of internal tagging, namely: Loher & Rensmeyer, (2011) assessed the physiological (healing, inflammation, infection; growth) effects and behavioural responses of Pacific halibut and also reviewed tag implantation techniques used in flatfishes; Fabrizio & Pessutti, (2007) evaluated tag retention and effects on survival, recovery and growth; while Moser *et al.*, (2005) analysed the short-term effects on survival and feeding behaviour. Other studies, with acoustic telemetry,

either briefly refer to unpublished results from captivity experiments where tagging effects or retention was evaluated (Fairchild *et al.*, 2009, 2013; Le Pichon *et al.*, 2014; DeCelles & Cadrin, 2010; Szedlmayer & Able, 1993), or simply omit information in this regard (Dance & Rooker, 2015; Furey *et al.*, 2013).

Moreover, studies that have marked *P. flesus* with acoustic tags give poor information on the effects of tagging in this species. Le Pichon *et al.*, (2014) reported a dismissal of data from the first week of field tracking flounder due to a delay in food intake, seen in a prior (but unpublished) captivity experiment; and Wirjoatmodjo & Pitcher, (1984) assessed tagging effects on frequency and distance of movements until 6 hours post-tagging only. Given the importance of collecting unbiased data for the reconstruction of movements of wild populations, research on the effects of tagging on the study species (or life stage) is essential and should be explored prior to performing field studies. Therefore, this study aimed to: (1) test two different tagging methods in *P. flesus*, one internal and one external; (2) assess the short-term effects of tag presence and attachment method on behaviour, physiological condition and survival; and finally (3) select the procedure with minimal negative effects and highest tag retention.

## 3.2 | Materials and methods

### 3.2.1 | Fish collection and quarantine

Flounder used in these experiments were caught in the lower Douro estuary (Figure 3.1) using an otter trawl from an outboard boat operated by a professional fisherman. As the quarantine system could only accommodate a maximum stock density at a time, fish were collected in different periods (table 3.1): large adults were caught in January 2017 (stock 1) and again in April (stock 2) as most of the first stock died over the course of the quarantine (see further details below and in chapter 4). Smaller sized flounder (stock 3) were collected in May 2017.

***Table 3.1 – Date of capture of each experimental stock of P. flesus; number of fish caught (N); and mean and range size (minimum – maximum) of fish length and weight for each stock. TL = Total Length; W = Weight; SD = Standard Deviation.***

| Sampling Date | Stock ID | N | Mean ± SD | | Size Range | |
|---|---|---|---|---|---|---|
| | | | TL (mm) | W (g) | TL (mm) | W (g) |
| January 18th, 2017 | 1 | 72 | 297±11 | 237±38 | 265 – 328 | 146 – 416 |
| April 19th, 2017 | 2 | 47 | 258±26 | 169±51 | 197 – 306 | 74 – 286 |
| May 24th, 2017 | 3 | 46 | 227±11 | 121±14 | 210 – 260 | 96 – 162 |

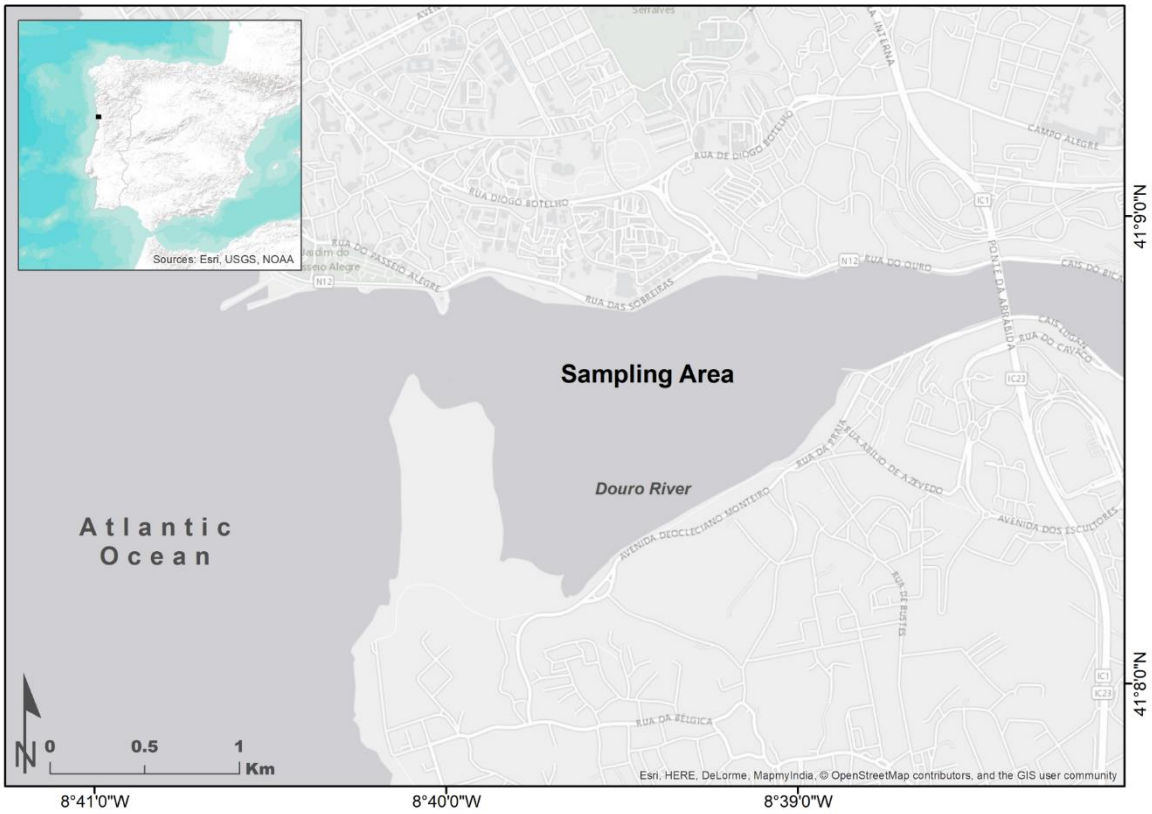


***Figure 3.1** – Map illustrating the area where flounder (P. flesus) were collected in the lower Douro estuary, Portugal.*

After capture, fish were immediately conditioned in aerated coolers with estuarine water and transported to BOGA, where they were held in a quarantine system (tank with 2200L, plus 500L in a *sump*) (figure 3.2) for a period of 57 days (stock 1), 25 days in the first actual experiment (stock 2) and 15 days in the second (stock 3). During the quarantine period, the water quality parameters (temperature, pH, salinity, ammonia, and nitrites) were monitored daily (see annexe). In all quarantine periods, there were feeding attempts with live fresh polychaetes with different responses depending on the stock: stock 1 did not feed until day 38, stock 2 fed after one week and stock 3 did not feed during the entire 2-week period that they remained in quarantine.

The tank was covered with a net or a plate to prevent fish from escaping (observed early in the quarantine period), and to try to reduce fish stress, given their acute escape response to outside movement close to the tank. The quarantine tank was kept without a sandy substrate to facilitate tank maintenance and the visual inspection of fish condition.

At the end of the quarantine period, the fish condition was visually evaluated and the fish that presented an overall good condition were transferred to the

experimental tanks (1800L).

***Figure 3.2*** *– System used during quarantine periods. Tank was either covered with a net or with a plate to provide shadow.*

Most of the first fish stock died of unknown causes associated with their capture and captivity or were euthanized due to poor condition. This was probably due to the proliferation of opportunistic agents associated with an immunodepression caused by the stress from acclimation to captive conditions; and by no food consumption over a period of more than a month. Microbiological analyses revealed water and tissue contamination by *Vibrio* spp. and *Pseudomonas* spp. (see Chapter 4), which could explain the deterioration of fish condition and heavy mortality.

Near the end of the established quarantine period flounder started to exhibit some skin ulcers with different degrees of severity. As the symptoms appeared the symptomatic flounder were isolated from the remaining fish stock, and transferred to a smaller (200L) and isolated tank. Because the fish kept developing ulcers, considering the fish welfare, and adding the fact that they could no longer be used in subsequent experiments where their behaviour and condition were critical to the results, the entire stock was euthanized using an overdose of 2-phenoxyethanol, following the ethical guidelines for fish welfare. Thus, further experiments were only conducted with stocks 2 and 3.

### 3.2.2 | Experimental set up and design

The two experimental tanks (figure 3.3), one for experiment 1 (stock 2) and

another for experiment 2 (stock 3), were kept under the same abiotic conditions as those used in the quarantine. The experimental tanks, however, had their bottom covered with a fine layer of sand to mimic the flounder's natural environment.

During the whole trial, the water quality parameters were monitored daily (figure 3.4 and annexe) and flounder were fed with live polychaetes every 2 to 3 days.

***Figure 3.3*** *– Experimental systems (1800L), used in experiment 1 and 2; covered with nets to provide shadow and with a sandy substrate.*

***Figure 3.4*** *– Instruments used to evaluate water quality daily: pH and temperature probe, refractometer and ammonia and nitrate reagents.*

In experiment 1 (hereafter called exp.1), only acoustic tags were used and the fish (n=30) were randomly distributed among 3 treatments: i) implant group (IG) composed of fish surgically implanted with transmitters; ii) external group (EG), where fish where marked with an external tag-mount; and iii) a control group (CG) where fish were handled but not marked. The CG was equally divided in two groups, a positive (with analgesic administration, C+) and a negative control group

(without analgesic administration, C-). In experiment 2 (exp.2), in addition to the acoustic transmitters also plastic tags (t-bar anchor tags) were tested. Flounder (n=26) were divided among 3 treatments: i) control group (positive and negative); ii) external group; and iii) a t-bar group (TG) which was equally divided between two groups with different tagging sites, in the dorsal musculature and between pterygiophores. The experimental design is shown in figure 3.5.

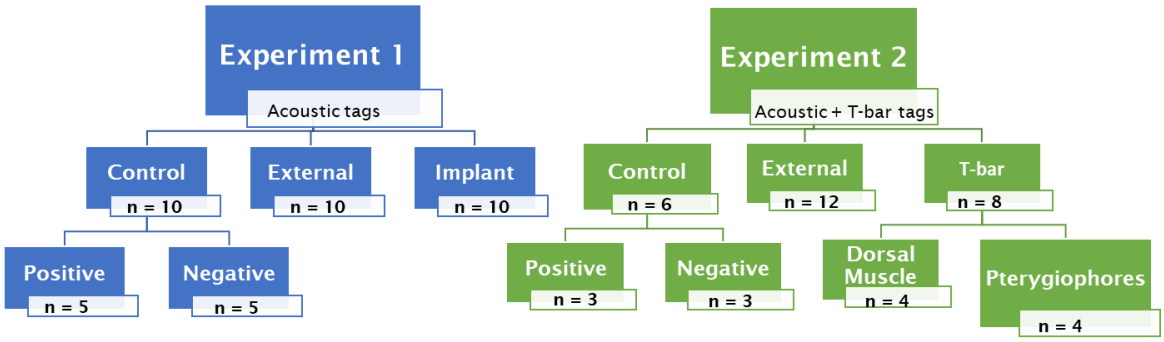


***Figure 3.5*** *– Schematic representation of the experimental design, describing the types of tags tested, treatments and the number of P. flesus (n) in each treatment.*

The two experiments lasted for 33 days and followed similar guidelines for maintenance, behavioural, and physiological observations.

### 3.2.3 | Tagging protocol

To minimise exposure and keep the procedures as aseptic as possible a few precautions were taken. The table was protected with a surgical drape, the operators used lab coats and swapped gloves often, all tags were disinfected as well as the PIT tag implanter needle, the fishing line, needle-holder, and forceps. All disinfections were done using a diluted Betadine® solution (10 mg/mL). The scalpel blades and sutures were sterile and discarded between fish.

One fish at a time was retrieved from the tank and placed in an anaesthetic solution (MS-222; 0.12g/L, [exp.1 – salinity 35 psu, 19˚C; exp.2 - salinity 36.4 psu, 20.4˚C]) with the help of a fish net. Fish remained in the induction bath for approximately 2 and a half minutes, the time needed to reach surgical anaesthesia (see Chapter 2), where a slower breathing rhythm was observed. Loss of equilibrium, the first sign of deeper stages of anaesthesia, was assessed by turning the fish with its uneyed side up and observing if the fish struggled to regain equilibrium or not.

Once unresponsive each fish's total length (TL) and weight were measured (figure 3.6) to the closest 1mm and 1g, respectively, and randomly distributed among the different treatment groups (table 3.2).

***Figure 3.6** – Flounder being weighed and measured.*

***Table 3.2 – Sample size (N), mean total length (TL) and mean weight (W) of fish within treatment groups in the two experiments. Tag burden (the ratio of transmitter weight to fish weight in air) was also calculated for each treatment. NA – Not applicable.***

| Exp. | Treat. | N | Mean ± SD | | Tag Burden (%) | |
|---|---|---|---|---|---|---|
| | | | TL (mm) | W (g) | Mean | Range |
| 1 | CG | 10 | 257 ± 26 | 168 ± 56 | NA | NA |
| 1 | EG | 10 | 261 ± 30 | 169 ± 60 | 1.6 | 1.0 – 2.7 |
| 1 | IG | 10 | 255 ± 36 | 168 ± 67 | 0.9 | 0.5 – 2.0 |
| 2 | CG | 6 | 229 ± 14 | 126 ± 19 | NA | NA |
| 2 | EG | 12 | 226 ± 15 | 120 ± 19 | 2.2 | 1.7 – 2.8 |
| 2 | TG | 8 | 226 ± 13 | 119 ± 16 | NA | NA |

Fish were then transferred to a surgical cradle where the remaining procedures were done under a maintenance anaesthesia (MS-222, 0.0375g/L), which was continuously provided by artificially ventilating the gills to keep fish anaesthetised to the surgical stage. Before starting the tagging procedures, flounder (with the exception of the negative control group and the t-bar group) were analgised with a lidocaine solution (Lidocaine 2%, B. Braun, 1mg/kg) delivered through injection.

In order to allow the identification of each individual throughout the experiment, each flounder was firstly tagged with a PIT (Passive Integrated Transponder) tag (FDX-B, 12x2.12mm, 88,9mg, Loligo®Systems), implanted in the posterior area of the peritoneal cavity (figure 3.7), with the aid of a disposable implanter that pierced the flounder's tegument on the eyed side. PIT tags are uniquely coded which allowed for each tagged to be individually identified by scanning them up close with a reader. At the beginning of the experiment the code of the PIT tag implanted in each of the fish was registered and at the end of the experiments fish were scanned and identified by the PIT tag code.

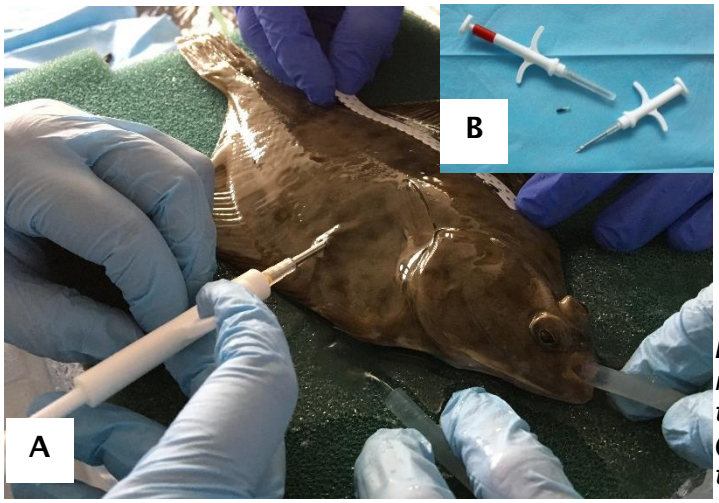


***Figure 3.7** – **(A)** Flounder being marked in the peritoneal cavity with the aid of a disposable implanter. **(B)** Disposable implanters and a PIT tag.*

3.2.3.1 | Control group

All flounder belonging to this group were subjected to the same general procedures (handling, anaesthesia, biometrics and marking with PIT tag) without any additional marking. They were kept in the surgical cradle for about 5 min, to assure that the time the fish were kept under anaesthesia was similar among treatments. The control group was further split to assess any effects of the lidocaine administration: the positive control group was given lidocaine while the negative group was not.

3.2.3.2 | Acoustic tagged groups

For testing the acoustic tagging methods, a dummy (inactive) tag from Vemco (model V7-2L, Vemco Ltd., Nova Scotia), was selected (figure 3.8). The V7 model was chosen to allow the tracking of smaller fish, and the 2L battery option for its better relationship between tag size and battery life (the smaller the tag the shorter the battery lifespan). The Vemco V7-2L tag measures 20 mm in length and

7 mm in diameter, and weights 0.75 g in water and 1.6 g in air. These specifications in combination with fish size were used to calculate tag burden, the ratio of transmitter weight to fish weight in air (table 3.2).

Fish were marked using two methods: (1) by externally attaching the tag to the fish dorsal musculature and (2) by intracoelomic implantation.

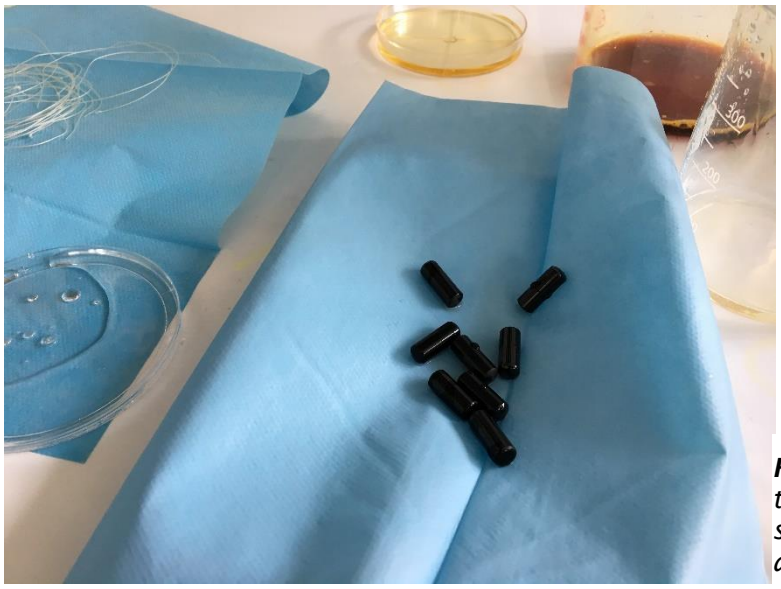

***Figure 3.8*** *– Several dummy tags (Vemco V7-2L) placed on a surgical drape after being disinfected.*

3.2.3.2.1 | Implant

This tagging method was performed only in the adult specimens (exp.1), as the peritoneal cavity of juvenile flatfish is too small to comfortably lodge an acoustic tag (Fairchild *et al.*, 2009).

The dummy tag, previously disinfected with a Betadine® solution, was introduced into the fish's peritoneal cavity through an incision of 1 to 2 cm, on the eyed side of the fish, between the pectoral and pelvic fins but posterior to the pectoral fin insertion, and perpendicular to the lateral line (adapted from Moser *et al.*, 2005 and Fabrizio & Pessutti, 2007). The tag was oriented parallel to the long axis of the fish body. The incision was closed with 2 individual sutures, double knotted, using Monosyn® sutures and a cutting needle (Monosyn® 4-0, B. Braun). The incision location was disinfected with Betadine® before the incision was made and after it was closed, and a local analgesic (lidocaine) was also applied to the area before making the incision (figure 3.9). The entire procedure (i.e. from entering the anaesthesia bath until entering the recovery bath) took approximately 6 and a half minutes for each fish. In this treatment, the PIT tag was inserted into the peritoneal cavity through the same incision as the acoustic tag instead of using

the disposable implanter. A single operator performed all the surgeries.

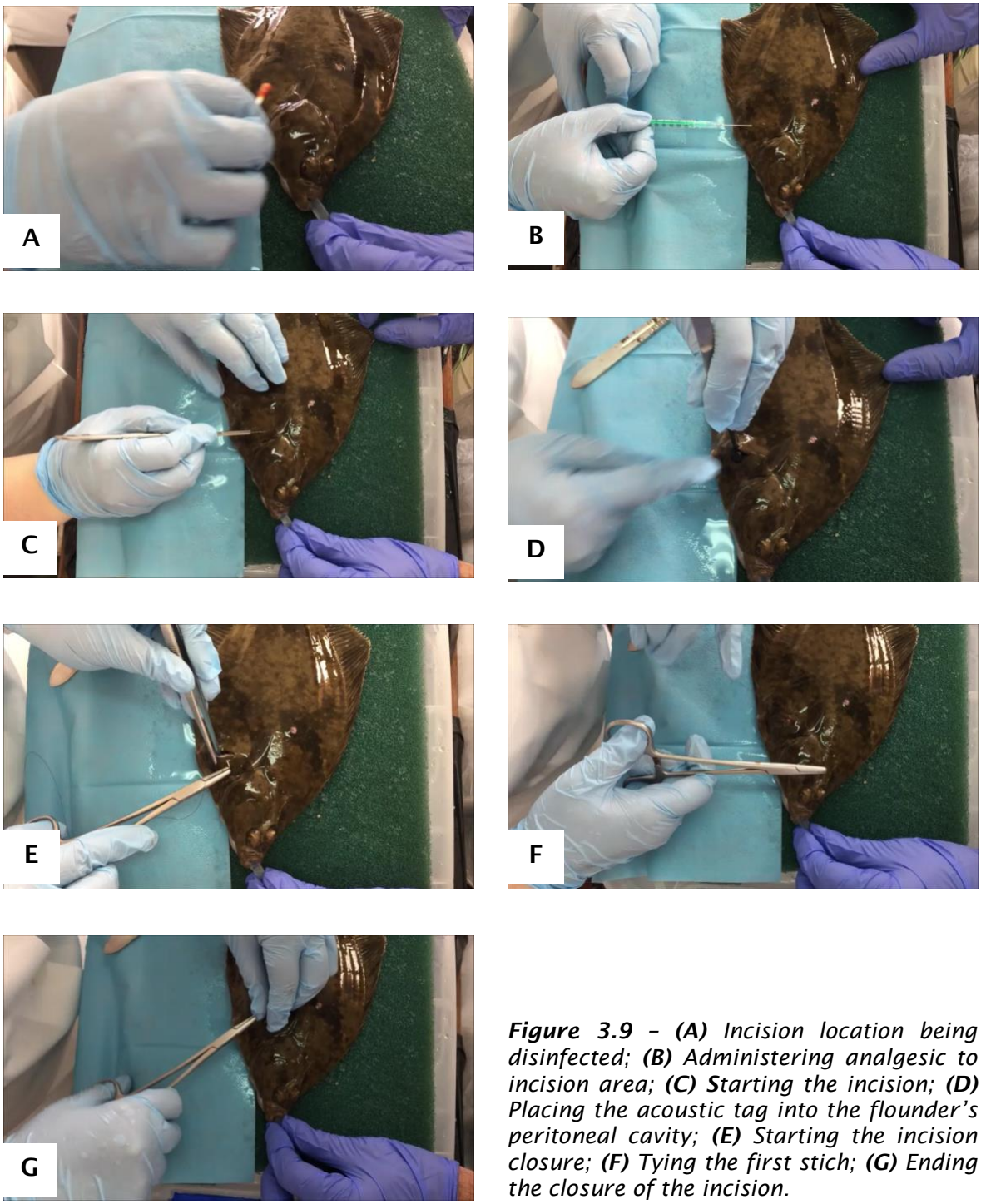


***Figure 3.9*** *– **(A)** Incision location being disinfected; **(B)** Administering analgesic to incision area; **(C)** **S**tarting the incision; **(D)** Placing the acoustic tag into the flounder's peritoneal cavity; **(E)** Starting the incision closure; **(F)** Tying the first stich; **(G)** Ending the closure of the incision.*

3.2.3.2.2 | External tag-mount

On the eyed and dorsal side of the fish, parallel to the pectoral fin, two hollow needles were inserted through the fish's musculature, from one side to the other. These needle insertion sites were previously disinfected with a Betadine® solution and analgised with lidocaine, and a plastic marker was used to insert the needles

at the same distance from one another. A fishing line (Berkley® Whiplash Crystal, braided, 0.21mm) was threaded through the first needle, from the eyed to the uneyed side; then through a silicone tube (used to protect the fishing line from constant abrasion with the bottom) and finally through the second needle, so both ends of the fishing line were on the eyed side of the fish. Each of the ends were then inserted through one side of a tube (compressor tube, 2.2cm). When each end of the fishing line was sticking out of each side of the tube a water pump plier was used to lodge the dummy tag inside the tube. Finally, the ends of the fishing line were knotted and heated on top of the tube (figure 3.10). The entire procedure (i.e. from entering the anaesthesia bath until entering the recovery bath) took approximately 8 min and 40 seconds for each fish, requiring a minimum of two operators.

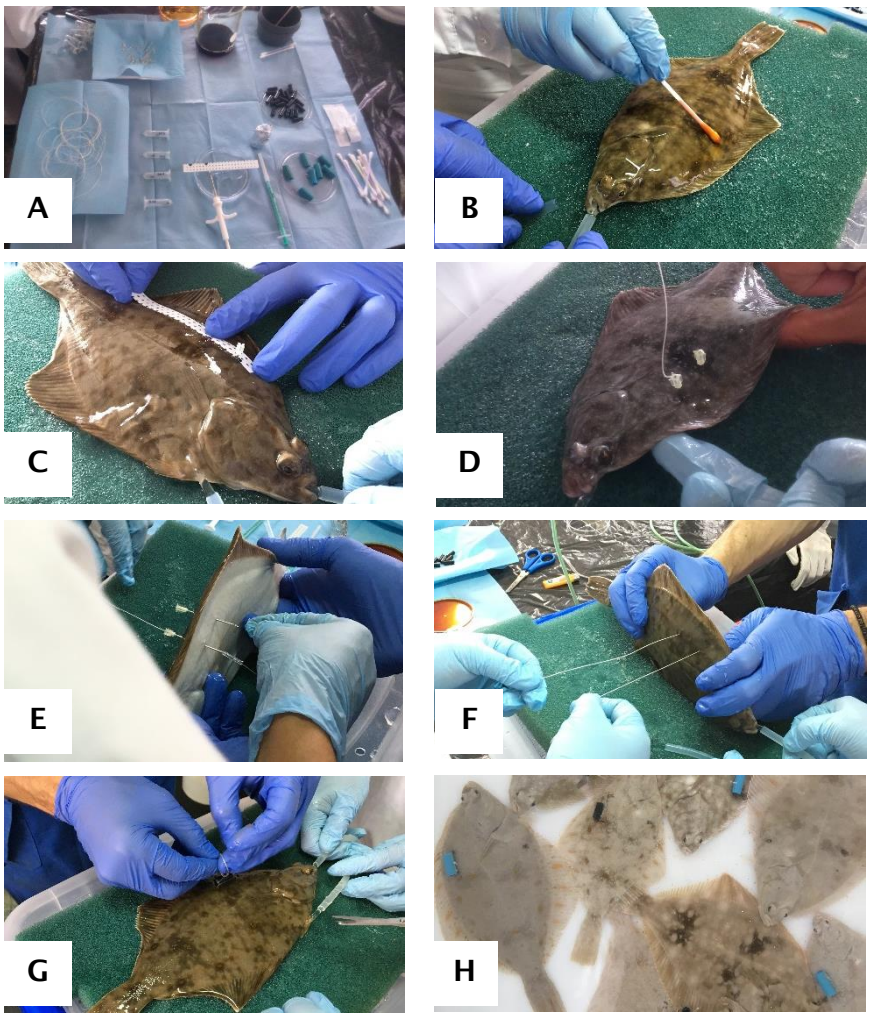


***Figure 3.10*** *– **(A)** All the materials used to externally mark the flounder; (B) Disinfection of the tagging site; **(C)** Plastic marker and insertion of the first hollow needle; **(D)** The two needles have been inserted and the first is being threaded; **(E)** Threading the second needle after having threaded the silicone tube; **(F)** Needles removed from the eyed side; **(G)** Tying the final knot on top of the external mount; **(H)** Tagged flounder in the recovery tank.*

3.2.3.3 | T-bar anchor tags

The tags chosen were the T-bar anchor tags types FF94 (0.106 g, 1 mm tubing diameter, 42 mm total length). These tags were applied with the tagging gun model Mark III fine fabric.

Fish were tagged with an individually numbered T-bar anchor tag; the tag was placed on the eyed side of the fish, either in the dorsal musculature near the head or between the pterygiophores of the dorsal fin (figure 3.11). The location of the tag was disinfected with a Betadine® solution prior to tag insertion.

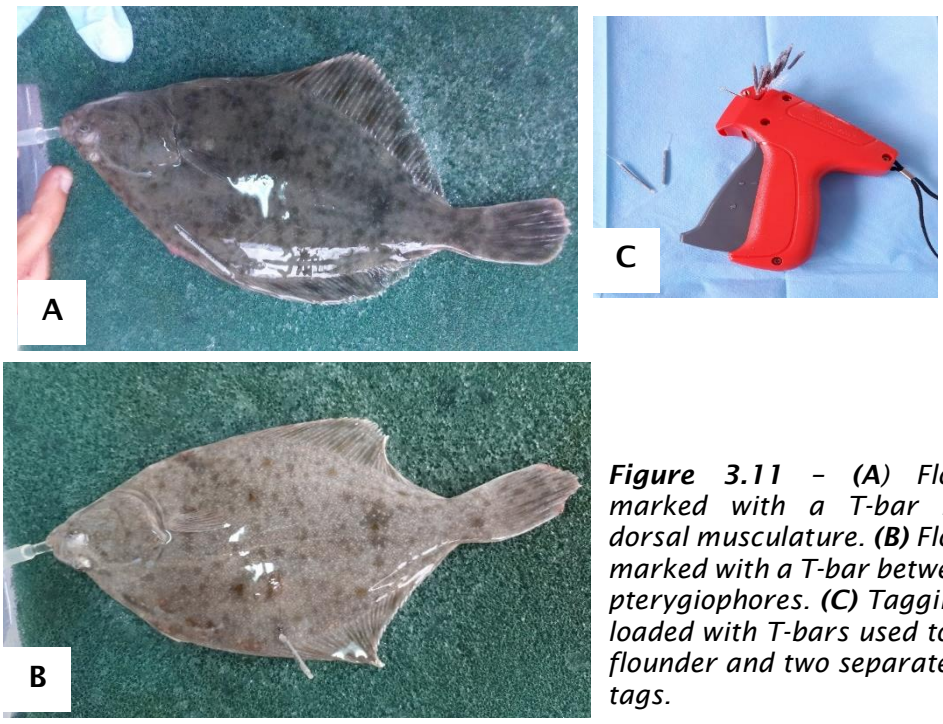


***Figure 3.11*** *– **(A)** Flounder marked with a T-bar in the dorsal musculature. **(B)** Flounder marked with a T-bar between the pterygiophores. **(C)** Tagging gun loaded with T-bars used to mark flounder and two separate T-bar tags.*

In all treatments, after completing all procedures, the still unresponsive flounder were transferred to a 200L recovery tank (figure 3.12) with highly aerated saltwater to recover from anaesthesia and analgesia. Only after they were completely recovered were they transferred to their experimental tank.

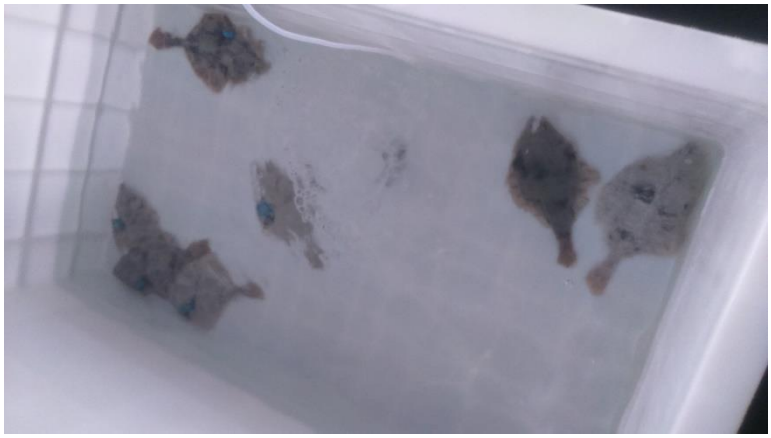

***Figure 3.12** – Flounder in recovery tank (200L) after being tagged.*

3.2.4 | Tag effect evaluation

3.2.4.1 | Behavioural observations

Over the course of the experiments flounder were observed to assess if the tagging procedures altered their normal behaviour. Video recordings were made on the days flounder were fed (every other day, 10 days in total) in two periods: before being fed (rest period), and during the feeding period that started when the polychaetes were introduced in the tank. In exp.1, feeding and behavioural observations started 4 days post tagging, and in exp.2, observations started after 3 days. The recording time (21 minutes) was the same for both periods, in each of the observational days.

For each experimental group, the frequency of each type of behaviour, according to the ethogram created (table 3.3), in the 21 minute period, was recorded.

***Table 3.3 – Behaviour ethogram for observations of Platichthys flesus.***

| Behaviour | Abbreviation | Definition |
|---|---|---|
| Swimming | Sw | Displacement of the body using body or fin movement as propulsion. |
| Biting | Bt | Fish captures a polychaete. |
| Scraping | Sc | Fish brushes itself against the walls or floor of the tank in an effort to dislodge the tag. |

3.2.4.2 | Physiological condition

At the end of each experiment all surviving flounder were euthanised with an overdose of 2-phenoxyethanol (1mL/L). All flounder were then measured and weighed; externally examined for lesions, inflammation (redness) and signs of infection (greenish hue of the skin protecting the internal organs); dissected for intra-abdominal examination of internal organs and area surrounding the tag. The tagged flounder final weights were corrected by subtracting the weight of the tag (plus the tag mount in EG) from the final body mass. Fish that died naturally or that were euthanized during the experimental period (fish from the IG with open incision wounds) were also measured, weighted and examined in similar ways.

At the end of the 33-day trials, survival, condition and growth were assessed.

The condition factor was calculated for each individual as

$$K = \frac{W_t}{W_e},$$

where $W_t$ is the fish's weight at time $t$ and $W_e$ is the theoretical weight of the fish calculated from the length-weight relationship estimated based on wild flounder population (own unpublished data):

$$W_e\ (g) = 0.0209\ TL^{2.818}.$$

At the end of the trial, the condition factor was only calculated for those fish who survived the 33-days period.

For fish that survived the entire experimental period, the specific growth rate (SGR, % day$^{-1}$) was also calculated:

$$SGR = \frac{log_e M_f - log_e M_i}{t} \times 100,$$

where $M_i$ and $M_f$ are the initial and final mass (g), respectively, and $t$ is the time in days.

The tanks were daily inspected for rejected tags. Tag retention rate was calculated, for both the acoustic and the plastic tag trials, at the end of both experiments. It was calculated as the number of retained tags divided by the total number of tagged fish multiplied by 100.

3.2.5 | Data analysis

Initial size, weight and condition of fish were compared among treatments, for each experiment, with a one-way analysis of variance (ANOVA).

The mean ± standard error for the three behaviours of each treatment group was calculated for both periods under analysis, and the results were compared across treatment groups in the same experiment. Moreover, differences in feeding

behaviour among treatments were evaluated using biting as a response variable while differences in flounder overall behaviour were analysed based on swimming data in the resting period. One-way ANOVA or non-parametric tests (Kruskal-Wallis) were used to statistically assess the effect of the tagging procedure.

Survival rate for each treatment was estimated using the Kaplan–Meier method (Kaplan & Meier, 1958) and differences in survival were examined using the log-rank test followed by pairwise comparisons when significant differences were detected.

Differences in final condition and in SGR among treatment groups were evaluated with a one-way analysis of variance (ANOVA), followed by Tukey HSD test for multiple comparisons if previous tests had significant results.

Statistical significance for all analyses was set at $\alpha = 0.05$, data were checked for normality and homogeneity assumptions, and analyses were performed with SPSS statistical package (SPSS v24.0).

## 3.3 | Results

### 3.3.1 | Initial fish size and condition among treatments

The initial condition factor of the fish in exp.2 was evaluated with a Kruskal-Wallis test because the data did not follow a normal distribution. In both experiments, there were no significant differences among treatments in either size (exp.1 – ANOVA; $F=0.106$, $p=0.900$; exp.2 - ANOVA; $F=0.114$, $p=0.893$), weight (exp.1 – ANOVA; $F=0.001$, $p=0.999$; exp.2 – ANOVA; $F=0.298$, $p=0.745$) or condition (exp.1 – ANOVA; $F=1.421$, $p=0.260$; exp.2 – Kruskal-Wallis; $\chi^2=1.953$, $p=0.377$).

### 3.3.2 | Survival

The overall survival rate was 43.3% in exp.1 and 61.5% in exp.2. The survival rate (table 3.4) in exp.1 was highest for EG and lowest for IG with significant differences among treatments (log-rank test, $\chi^2=17.134$, $df=2$, $p<0.05$). In this case, after pairwise comparisons, it could be seen that the survival of the implant group (IG) was significantly lower than both control and external groups (log-rank test, $\chi^2_{IG,\,CG}=6.704$, $p<0.05$; log-rank test, $\chi^2_{IG,\,EG}=12.790$, $p<0.05$). In exp.2, the EG experienced a lower rate of survival compared to TG and CG but no significant differences in survival were observed among groups (log-rank test, $\chi^2=3.192$, $df=2$, $p=0.203$).

The average time the fish in each treatment survived is shown in table 3.4. In exp.1 EG was the treatment where fish survived the longest (30.3 days) while fish

in IG only survived on average 12.2 days; in exp.2 the average time of survival was very close among all treatments, the shortest being 27.7 days in CG and the longest 29.4 days in TG. Figures 3.13 and 3.14 illustrate the probability of survival in each experiment for each treatment at any time of the experiment and indicates when each fish died.

***Table 3.4 – Number (N) of surviving flounder at the end of the 33-days trials, for each treatment in each experiment, along with the survival rate, the average time fish in each treatment survived and the percent growth rate. (*) – only one fish survived. CG = Control group; EG = External group; IG = Implant group; TG = T-Bar group.***

| Exp. | Treat. | N | Survival (%) | Days of Survival (Mean ± SD) | % SGR (Mean ± SD) |
|---|---|---|---|---|---|
| 1 | CG | 4 | 40.0 | 26.1 ± 9.1 | -0.56 ± 0.43 |
| 1 | EG | 8 | 80.0 | 30.3 ± 6.5 | -0.49 ± 0.30 |
| 1 | IG | 1 | 10.0 | 12.2 ± 7.7 | * |
| 2 | CG | 4 | 66.7 | 27.7 ± 9.4 | -0.10 ± 0.22 |
| 2 | EG | 5 | 41.7 | 28.8 ± 5.1 | -0.50 ± 0.19 |
| 2 | TG | 7 | 87.5 | 29.4 ± 10.3 | -0.43 ± 0.26 |

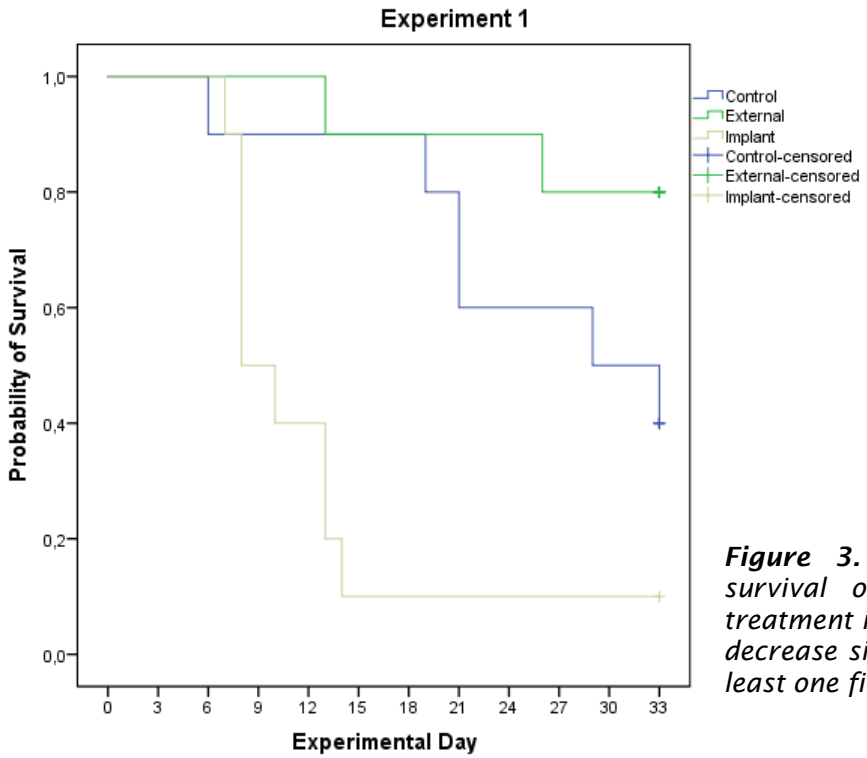


***Figure 3.13** – Probability of survival of flounder in each treatment in experiment 1. Each decrease signals the death of at least one fish.*

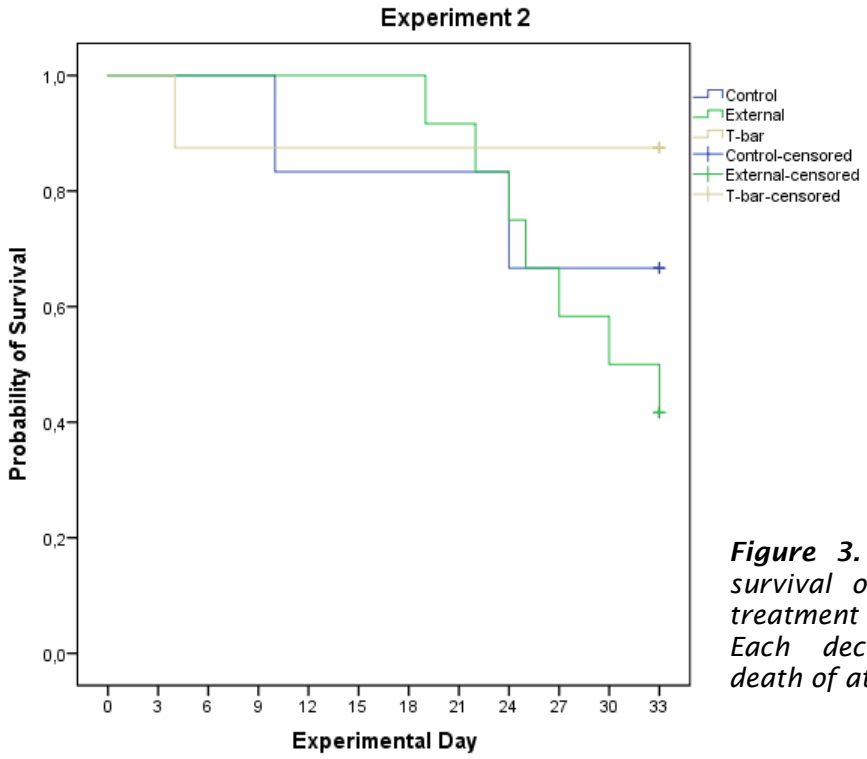


***Figure 3.14*** *– Probability of survival of flounder in each treatment in experiment 2. Each decrease signals the death of at least one fish.*

In both experiments, the survival of the C+ group was greater than the survival of the C- group, in number of surviving specimens and on average time of survival (exp.1 – C+: 60.0%, 29.4±6.1 days; and C-: 20.0%, 22.8±11.1 days; exp.2 – C+: 100.0%, 33.0±0.0 days; and C-: 33.3%, 22.3±11.6 days). Survival of fish tagged with T-bars was similar between the two marking locations: TG dorsal muscle: 75.0%, 25.8±14.5 days; and TG pterygiophores: 100.0%, 33.0±0.0 days.

Tag retention was 100% for all the treatments, except for the TG (87.5%) where an individual tagged in the dorsal musculature shed its tag. Moreover, the retention of the PIT tag was also 100% in both experiments.

### 3.3.3 | Condition and growth

At the end of exp.1, only flounder from the EG showed signs of inflammation, near the tagging wounds and mostly on the uneyed side while wounds on the eyed side had already healed (figure 3.15). The one individual from the IG who survived the whole experimental period still had closed sutures and the wound had healed (figure 3.15). In exp.2, inflammation was observed in both EG (also greater on the uneyed side) and TG treatments. However, in the TG, inflammation was only observed in flounder tagged between the pterygiophores (figure 3.15). Signs of

infection were more frequent in exp.1 with fish from the 3 treatments exhibiting infection of tissues to some degree while in exp.2 only one flounder from EG was affected. In both experiments two fish from the EG developed wounds related to the tag, some even severe, on the dorsal musculature near the tag placement. External examination of all flounder also revealed the development of skin ulcers in most of them (this is further explored in Chapter 4). In the IG, most had the sutured incision partially, if not totally, open (figure 3.15), and some fish expelled part of the digestive tract through the incision. One fish from the EG, in each of the experiments, was emaciated. Fish from both experiments also showed a build up of fluid in the peritoneal cavity. The aspect of the liver was not always the same: some were paler than normal (n=6) observed in at least one fish per treatment; blackish livers were also found in some cases (n=4) but only on those fish that died naturally over the course of the experimental period.

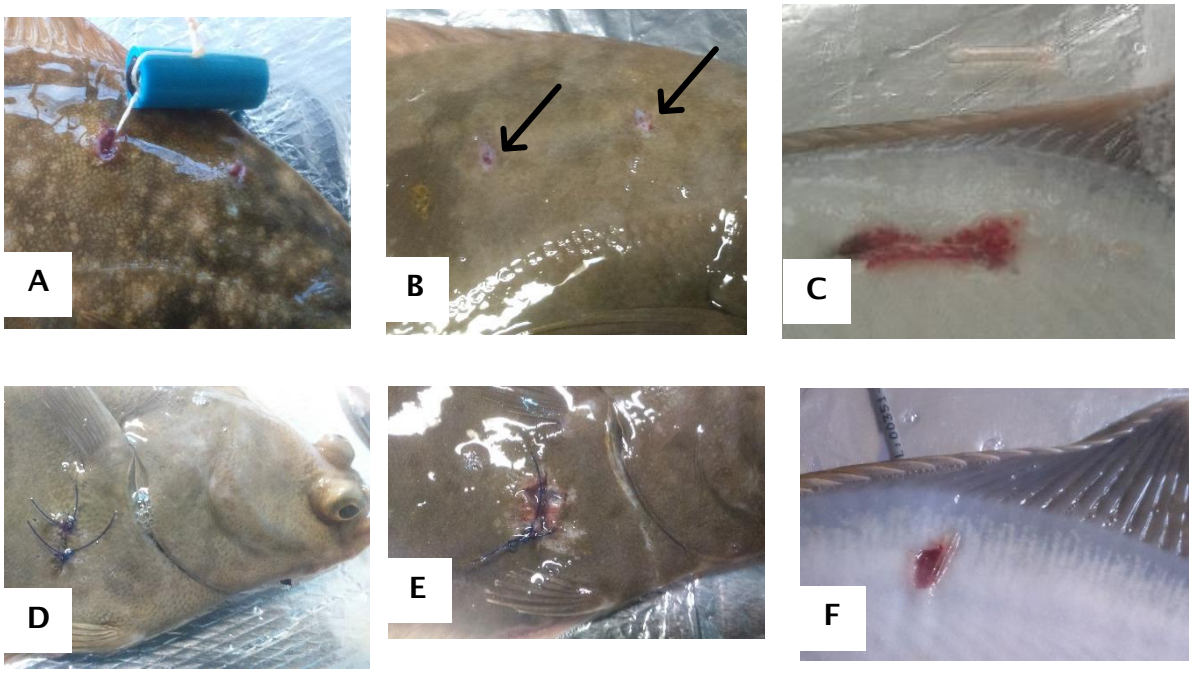


***Figure 3.15*** *– **(A)** Inflamed tagging sites on the eyed side; **(B)** Healing tagging sites on the eyed side, arrows point towards the locations where the needles were inserted during the tagging procedure; **(C)** Greatly inflamed uneyed side of externally tagged flounder; **(D)** Healing suture; **(E)** Open sutures and open incision wound; **(F)** Inflamed T-bar tagging site (between the pterygiophores).*

Fish condition decreased from the beginning to the end of the trials in both experiments (table 3.5). However, differences among treatments were only significant in exp.1 (ANOVA; $F=5.441$, $p<0.05$), with the EG exhibiting worse condition than the CG. In exp.2 fish from the CG had a higher final condition factor but there were no significant differences among treatments.

***Table 3.5 – Fish condition factor at the beginning ($K_i$) and at the end ($K_f$) of the trial for all treatments. (*) – only 1 fish survived. CG = Control group; EG = External group; IG = Implant group; TG = T-Bar group.***

| Exp. | Treat. | $K_i$ | | $K_f$ | |
|---|---|---|---|---|---|
| | | Mean ± SD | Range | Mean ± SD | Range |
| 1 | CG | 0.83 ± 0.06 | 0.75 – 0.90 | 0.75 ± 0.10 | 0.59 – 0.88 |
| 1 | EG | 0.79 ± 0.07 | 0.66 – 0.90 | 0.68 ± 0.08 | 0.56 – 0.82 |
| 1 | IG | 0.83 ± 0.06 | 0.71 – 0.89 | * | * |
| 2 | CG | 0.89 ± 0.06 | 0.77 – 0.96 | 0.83 ± 0.05 | 0.77 – 0.86 |
| 2 | EG | 0.88 ± 0.05 | 0.79 – 0.94 | 0.74 ± 0.05 | 0.66 – 0.80 |
| 2 | TG | 0.86 ± 0.06 | 0.81 – 0.99 | 0.77 ± 0.08 | 0.63 – 0.90 |

At the end of the 33-days of both experiments, SGR was negative for all treatments (figure 3.16). For the IG, SGR was not calculated because only one fish survived the entire period. While in exp.1 there were no differences in SGR among treatments (ANOVA; $F=0.111$, $p=0.746$), in exp.2 the mass loss was on average larger in tagged fish compared to the control (ANOVA; $F=3.856$, $p<0.05$) and differences in mass loss were statistically significant between the CG and EG (Tukey HSD; $p<0.05$).

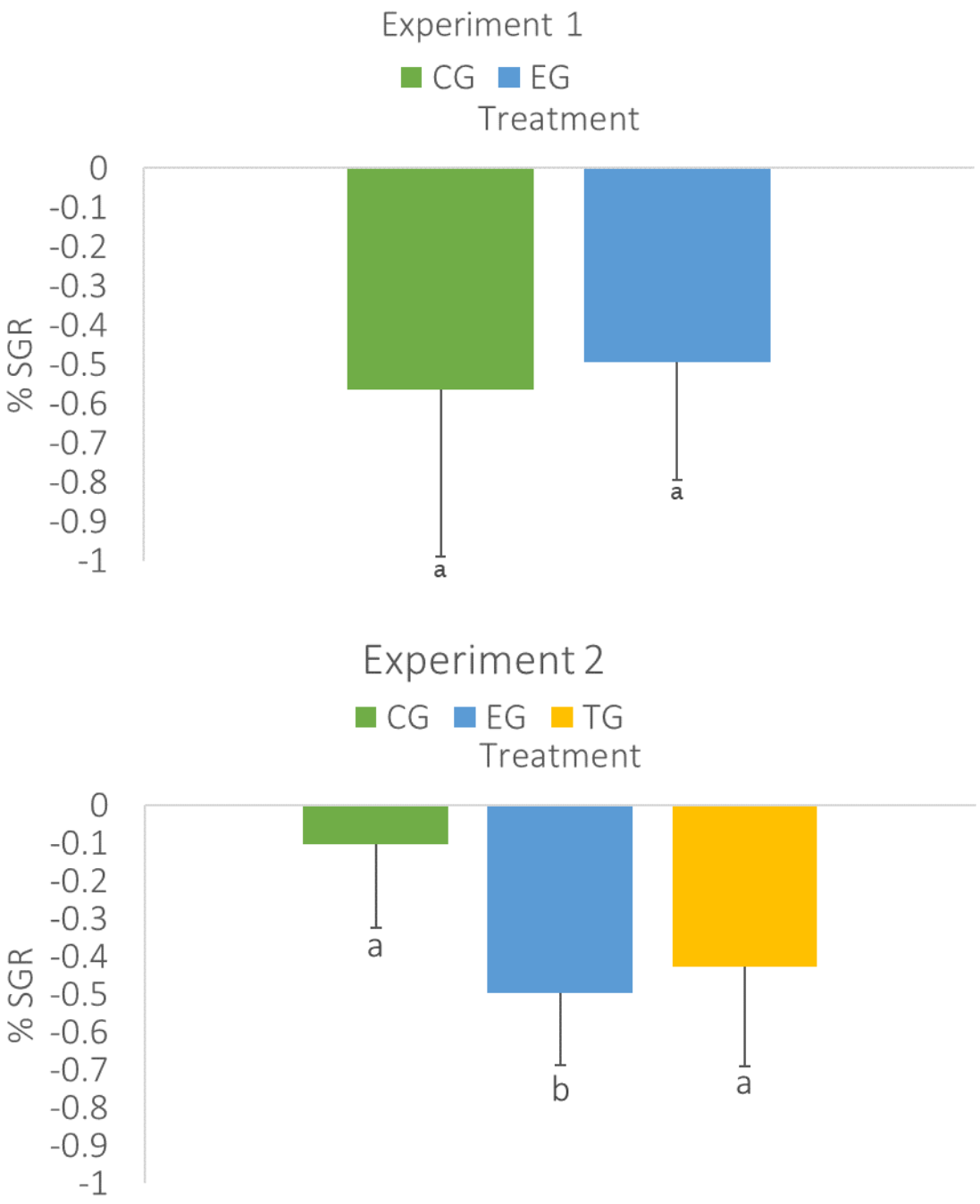


***Figure 3.16 – Specific growth rate of P. flesus in the various treatment groups in experiment 1 and 2.*** *Data calculated as mean % SGR ± SD. Sample sizes are different between treatments because of mortality. In exp.1 n = 4 and n = 8 respectively for control and external treatment groups. In exp.2 n = 4, n = 6 and n = 7 respectively for control, external and T-bar treatment groups. Within each experiment, bars not sharing the same letter are significantly different at p < 0.05 (Tukey HSD).*

### 3.3.4 | Behavioural observations

Throughout both experiments the scraping behaviour was never observed, suggesting that flounder did not actively try to remove the tags.

The mean frequency and standard error of each of the other observed behaviours, during the 10 observation days, per treatment and observation period (resting and feeding) can be seen in table 3.6. As expected the biting behaviour was only observed in the feeding period, with the exception of one flounder (from EG) that exhibited this behaviour once during resting. The swimming behaviour was observed more frequently during the feeding period reflecting the hunt/search for prey. In exp.1, fish from EG swam and ate the most during the feeding period, while in the resting period fish from CG were the most active. However, these differences in behaviour did not statistically vary between treatments (EG, CG) (feeding: ANOVA; $F=0.150$, $p=0.703$; resting: ANOVA; $F=2.278$, $p=0.116$). Due to the high mortality of the IG, behavioural observations of this treatment stopped when 6 individuals had died. Therefore, the behaviours of the IG were only studied in the first two observational days, justifying its low values and the exclusion of this treatment group from statistical analysis.

In exp.2 during the feeding period the TG was the group that fed and moved the least. However, this situation was reversed in the resting period with fish from TG showing higher activity (table 3.6). Similarly to exp.1, no statistical differences among treatments were observed (feeding: ANOVA; $F=2.869$, $p=0.074$; resting: KW; $\chi^2=1.157$, $p=0.561$).

***Table 3.6 – Mean and standard error of the frequency of all the observed behaviours, in each observation period for each treatment, in both experiments. The scraping behaviour is not included because it was never observed. Exp. = Experiment; Treat = Treatment group; SW = Swimming; BT = Biting; CG = Control group; EG = External group; IG = Implant group; TG = T-Bar group.***

| Exp. | Period | Treat. | Mean ± SE | |
|---|---|---|---|---|
| | | | SW | BT |
| 1 | Feeding | CG | 23.1 ± 4.6 | 12.3 ± 2.6 |
| | | EG | 25.8 ± 2.4 | 13.5 ± 1.8 |
| | | IG | 4.0 ± 2.0 | 0.0 ± 0.0 |
| | Resting | CG | 19.7 ± 3.0 | 0.0 ± 0.0 |
| | | EG | 13.1 ± 2.7 | 0.1 ± 0.1 |
| | | IG | 0.5 ± 0.5 | 0.0 ± 0.0 |
| 2 | Feeding | CG | 18.0 ± 2.3 | 10.8 ± 1.3 |
| | | EG | 18.5 ± 4.0 | 8.8 ± 1.3 |
| | | TG | 11.6 ± 2.8 | 6.5 ± 1.2 |
| | Resting | CG | 6.9 ± 1.9 | 0.0 ± 0.0 |
| | | EG | 4.9 ± 2.3 | 0.0 ± 0.0 |
| | | TG | 9.0 ± 3.1 | 0.0 ± 0.0 |

In figures 3.17 and 3.18 it's possible to see the percentage of the biting behaviours (figure 3.17) and of swimming behaviours (figure 3.18) in the feeding and resting period, respectively, throughout the 10 observation days for each of the treatments. The number of counted behaviours was corrected for the number of fish alive in each of the observational days in each of the treatments.

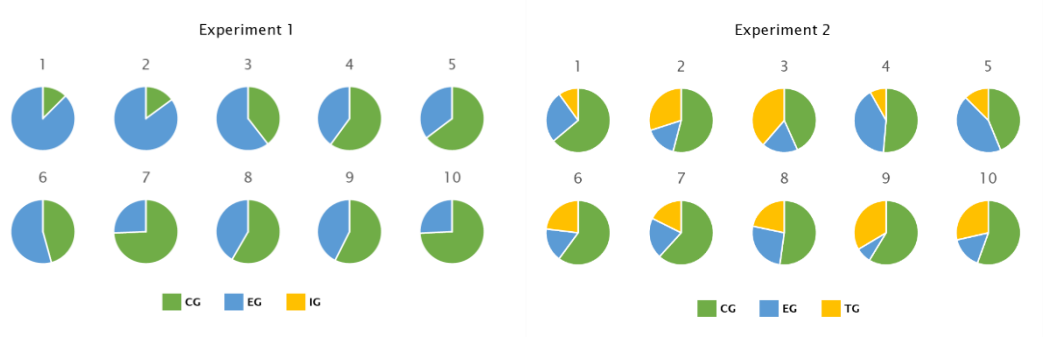


***Figure 3.17 – Percentage of biting behaviour, observed during the feeding period, made by fish of each treatment and corrected for the number of fish alive in each treatment in each of the observational days.*** *Green and blue represent control and external treatments in both experiment 1 and 2; yellow represents the implant group in experiment 1 and the T-Bar group in experiment 2; numbers represent, in order, each of the 10 observational days.*

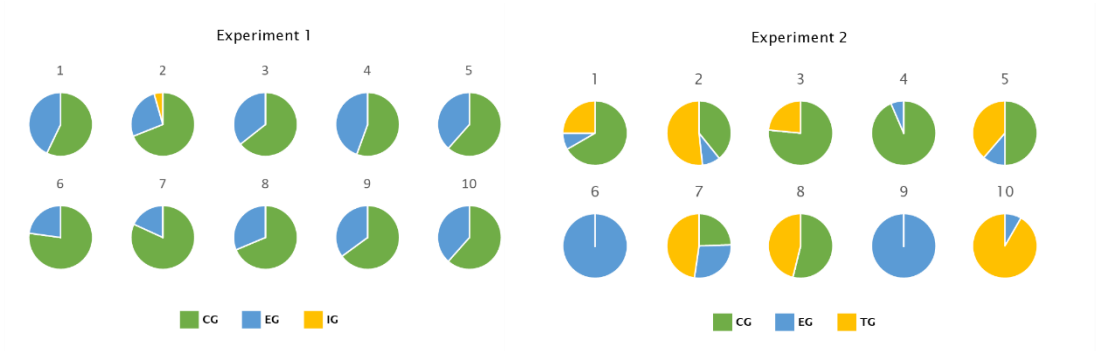


***Figure 3.18 – Percentage of swimming behaviour, observed during the resting period, made by fish of each treatment and corrected for the number of fish alive in each treatment in each of the observational days.*** *Green and blue represent control and external treatments in both experiment 1 and 2; yellow represents the implant group in experiment 1 and the T-Bar group in experiment 2; numbers represent, in order, each of the 10 observational days.*

The number of fish that fed throughout the experiments was not constant. In exp.1 the number of feeding fish in an observational day varied between 3 and 8 fish, and on average only 6 fish ate in each of the days; in exp.2 it varied between 5 and 12 fish, with an average of 8 feeding in each of the days. Figure 3.19 shows the time since the introduction of the food into the system until the time when the first fish from each treatment ate. Usually a fish from the control group was the first to eat, and that was common to both experiments. In exp.1 the feeding behaviour between CG and EG was quite different at least until the seventh observation day, and the delay between the EG and the CG feeding time was approximately 0.2 min. While in exp.2, the feeding behaviour followed a similar

temporal pattern throughout most of the 10 observational days but with a delay among treatments, with the EG typically being the last to start eating (EG takes approximately 2.7 min more to start eating than the CG; and the TG takes approximately 2.4 min more).

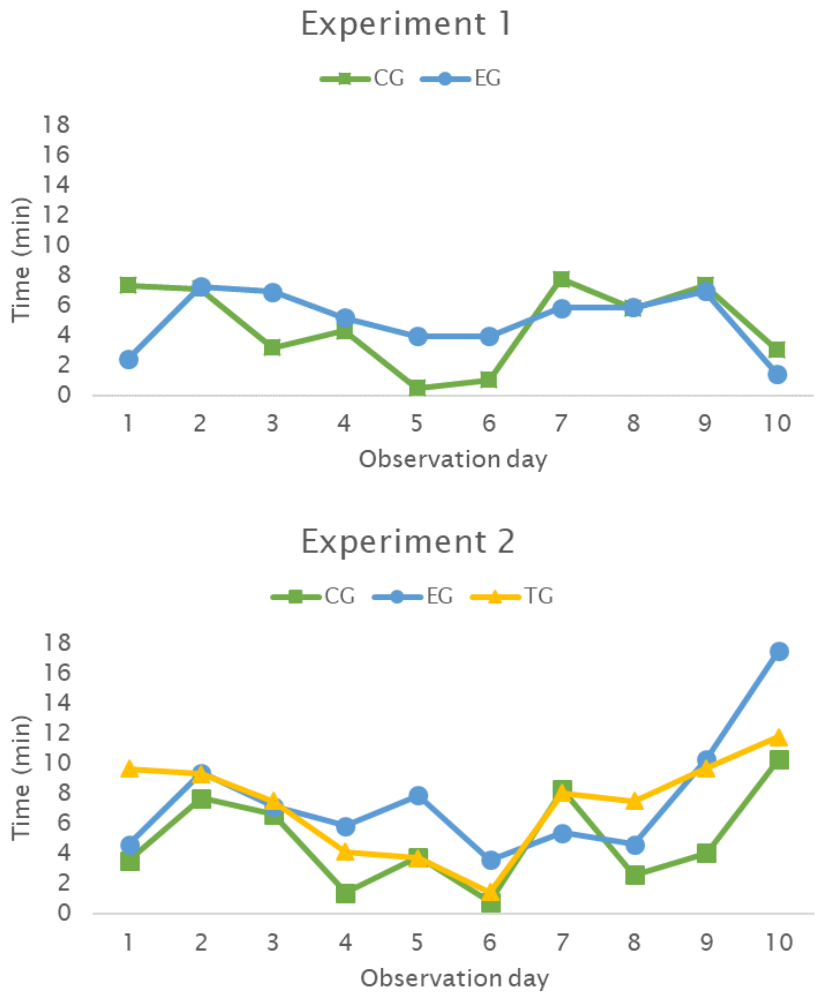


***Figure 3.19*** *– Time, in minutes, taken since the entry of food in the system until the first bite by a flounder from each treatment in each of the observational days.*

## 3.4 | Discussion

Our results suggest that maintaining *P. flesus* in captivity is a challenge. The overall mortality including the two experiments was about 50%, regardless of treatment. This high mortality rate suggests that the conditions in which flounder are held need to be optimized so that one can disentangle the captivity from tagging effects.

We concluded that the most invasive procedure for tagging *P. flesus* was the intracoelomic implant. The survival of the implant treatment (10%) was clearly affected, probably not directly due to the tag but because of the reopening of the incision, i.e. loosening of the suture knots, making the fish more vulnerable to diseases. Moreover, the first death in this treatment occurred 7 days after surgery, reinforcing that it was not the tagging procedure itself that was harmful but the failure in properly closing the incision. It is also important to say that with a more experienced surgeon the incisions would probably remain closed having more time to heal so that the impacts of this procedure on fish survival would not be as accentuated as they were in this study. Previous works where flatfish were also internally marked, such as Moser *et al.* (2005) and Fabrizio & Pessutti (2007) incision closure was not an issue because incisions typically healed completely. In those studies, the reported mortality rate was much lower and mostly due to lesions caused to the internal organs during surgical procedures. However, necropsies done on flounder from this study showed that no damage occurred during the surgical procedures. Moreover, there were no signs of intraperitoneal fibrous adhesions to the incision (as seen in Moser *et al*., 2005) and neither were there any other signs of tag encapsulation. Tag encapsulation, by opaque fibrous tissue (Moser *et al*., 2005) or even calcitic material (Loher & Rensmeyer, 2011), is sometimes a precursor to tag expulsion; the tag is completely enveloped by intestinal mesenteric tissue and then expelled. In this study tag encapsulation, even partial, was not observed and the tag remained free in the peritoneal cavity. Although most individuals from the implant group might have died before this could happen, this also did not happen with the PIT tags in any flounder.

Generally, the external attachment procedure was more effective with a lower impact on fish and thus allowing a quicker recovery. Fish from this group, for instance, ate readily on the first observation day in contrast to fish from the implant group. This has also been observed by Pursche *et al.* (2014) where the onset of post-tagging feeding of internally tagged fish happened several days later than externally marked fish. In terms of survival this treatment resulted in

substantial mortality, which was also observed in the control group and therefore we cannot conclude that the tagging affected survivorship. However, we observed that the mortality rate among the two experiments was different with the smaller sized flounder (exp.2) suffering from higher mortality. It remains unclear whether size could explain this differential effect. Further experiments with different sized fish groups would be necessary to test this feature.

In this study we also assessed if the external mount caused damage on the fish dorsal musculature. Fish reaction to the external mount was similar between both experiments, at the end of the 33-days trials inflammation was always greater on the uneyed side than on the eyed side. However, most fish still had slightly inflamed wounds on the eyed side and very few were totally healed. The location of the lesions on the eyed side suggests that the mount may be too tight, however loosening it may cause additional drag (Jepsen *et al.*, 2015) or result in a sawing motion as the fish swims, causing the line to cut into the fish. The external mount procedure can still be further improved by either not applying the thinner tube on the uneyed side or by using another material to attach the tag to the fish body.

Other observed changes to flounder normal condition, such as liver abnormal aspect and the build up of fluid in the peritoneal cavity, did not seem related to tagging experiences, because in a parallel study, wild flounder from Douro estuary were found with the same conditions (personal observations).

The fish's growth in mass and final condition was impaired in all treatments. Growth of the tagged groups did not vary in any of the experiments. However, in exp.2 there was a tendency for larger loss of mass of the marked compared to the unmarked fish. Previous works (Bégout Anras *et al.*, 2003, Larsen *et al.*, 2013 and Smircich and Kelly, 2014) have reported a negative effect on growth after tagging that is later compensated, however the duration of the present experiments (33 days) was relatively short. It is thus possible that the energy initially spent on adapting to the tag, which was seen as negative growth rate, had not still been compensated, raising doubt if fish would recuperate if the experiment had been longer. Moreover, the loss of mass in the control group also suggests that rearing conditions and the stress of the experimental procedures might have affected growth.

Procedures associated with the external attachment of tags to fish do not seem to have an immediate effect (within the initial days of the trials), or even a later effect, on this species' feeding or swimming behaviour. Fish from the control group usually responded quicker to the food stimulus. It is unclear whether this is a

consequence of a linear hierarchy on flounder feeding behaviour where the dominant fish always feeds first, as it was not possible to visually single out each fish within a treatment group. Flatfish are generally considered non-aggressive fish (Carter & Davies, 2004; Fatsini *et al.*, 2017) but in a recent study with Senegalese sole dominance categories with associated behaviours have been identified (Fatsini *et al.*, 2017) suggesting a feeding dominance ranking.

It was expected that towards the end of the experiment, fish would have learned that the introduction of the video camera into the system meant that they were going to be fed and so the response to the food would be quicker, however this was not observed. Overall, there were no differences in the feeding behaviour among treatments, but nevertheless the external treatment response time to food in exp.1 was similar to the control group in the last observational days. The same did not happen in exp.2, where the temporal pattern of response to food was similar among treatment groups but the delay between treatments is highly variable.

The external tagging procedure has the least impacts on fish condition, survival and behaviour. However, considering the difficulties associated with holding flounder in captivity it would be advisable to perform a similar experiment but this time in a field setting, keeping fish in enclosures. In addition, such an experimental setting would allow testing other factors namely of tag entanglement, that are more likely to occur in a natural system.

# Chapter 4 | Development of skin ulcerations in wild *Platichthys flesus* held in captivity

## 4.1 | Introduction

Skin ulcer disease is a frequent pathology in several fish species and a well-recognized indicator of stressed aquatic environments (Noga, 2000). Fish skin is metabolically very active and quickly responds to stressors; the intimate relationship between fish skin and the surrounding environment explains, at least partially, why epidermal damage is used as a biomarker of stressful environments (Noga, 2000).

Macroscopically, the ulcers' development seems to start as small circular haemorrhagic lesions (surrounded by a whitish border) and shedding of epidermis exposing the underlying musculature without, usually, penetrating deeply into it (Wiklund & Bylund, 1993). The causes of this disease have been attributed to several possible etiological factors: overcrowding; pollution; large salinity fluctuations; low condition factor due to shortage of food; ammonia; pH extremes, and temperature (Wiklund & Bylund, 1993; Noga, 2000).

In wild fish, although not clearly determined if caused by pollution, salinity and injuries from fishing gears seem to partially explain the prevalence of skin lesions. However, host-related factors (age, sex and length) also influence the prevalence of this pathology (Wiklund & Bylund, 1993; Vethaak & Jol, 1996). In aquaculture, this disease is usually associated with septicaemic conditions caused by bacteria of the genera *Vibrio*, and gram negative *Aeromonas* and *Pseudomonas* (Wiklund & Bylund, 1993), without affecting the internal organs (Devesa *et al.*, 1989). As these bacteria are opportunistic and ubiquitous, in marine and brackish waters, they probably act as secondary invaders and are likely not the primary cause for the disease. They can be present, in smaller numbers, in healthy skin allowing them to quickly colonize wounds caused by any trauma to this protective layer (Noga, 2000). Stress lowers disease resistance, making the individuals more vulnerable to infections.

Flatfish (Pleuronectiformes) appear to be one of the most vulnerable fish groups to these ulcerative skin lesions (Wiklund & Bylund, 1993).

In this study, we report on the development of skin ulcerations in flounder *Platichthys flesus* collected in the Douro estuary and kept under captive conditions.

## 4.2 | Material and methods

### 4.2.1 | Experimental fish

On January 18th, 2017, seventy-two flounder (size range: 26.5-32.8 cm TL, 146-416 g) (stock1) were captured in the lower Douro estuary. These individuals were caught with the help of a professional fisherman and an otter trawl, in three consecutive trawls, of approximately 15 minutes each, in different areas of the lower estuary.

After capture, flounder were conditioned in two aerated coolers with estuarine water, and quickly transported to BOGA, where they were promptly transferred to a 2200L tank with a 500L *sump*. Fish remained in a quarantine for a period of 57 days because they started to present some severe skin ulcers. At the end of the 57 days the symptomatic fish were euthanised with an overdose of anaesthetic (2-phenoxyethanol, 1mL/L). The remaining asymptomatic fish (n=18) were transferred to a new system for 21 days at the end of which they were also euthanized with an overdose of anaesthetic. These 18 flounder were kept in captivity for a total period of 78 days. There were some feeding attempts, but flounder only started to respond to the food on day 38. After that, flounder were fed every 2 to 3 days with fresh polychaetes.

The following stocks, 2 and 3, were caught, respectively, on April 19th and May 24th, 2017. The capture and transport procedures were the same as those performed with stock 1, as well as the captivity conditions. Stock 2 was composed of 47 flounder (size range: 19.7-30.7 cm TL, 74-292 g), which started to feed on the 8th day of confinement, and they remained in the quarantine tank for a period of 32 days. They were then transferred to the experimental tank where they remained for 33 days. The third stock was composed of 46 flounder (size range: 21.0-26.0 cm TL, 96-162 g) and remained in the quarantine tank for a period of 14 days, without feeding. They were then transferred to the experimental tank where they remained for another 33 days. Flounder from stock 2 and 3 that survived the whole captivity period remained in captivity a total of 65 and 47 days, respectively, at the end of which they were euthanised with an anaesthetic overdose. After the quarantine period both stocks ate fresh polychaetes regularly (every other day).

### 4.2.2 | Microbiological sampling and processing

Symptomatic flounder (n=2) from stock 1 were collected from the isolation tank and euthanised to perform microbial analysis. Fish were euthanized by severing the medulla oblongata because the use of anaesthetics could induce changes in the microbial community.

Material from the skin ulcers and from the kidney was swabbed and then streaked onto different agar mediums, to determine which bacteria were present. Water from the system was also collected and analysed to see if there were any pathogens present.

The overall condition of the two individuals was also macroscopically examined and their total length measured.

### 4.2.3 | Examination

Before starting flounder examination, all flounder (except those used for microbial analysis) were collected from the tank and euthanised with an anaesthetic (2-phenoxyethanol, 1 mL/L) overdose, and then weighed and measured (total length).

Flounder were inspected externally and internally. External inspections started with the careful observation of the surface of the flounder on both body sides, including head, gills and fins, and recording the presence of any ulcers/skin lesions and parasites. The internal examination was to assess and register the aspect of the internal organs, digestive tract and liver, the accumulation of liquid in the peritoneal cavity, identify the maturity stage and sex (in case of adults) and the presence of internal parasites.

The presence of ulcers was recorded according to four areas: head (eyed and uneyed side, from mouth to gill opening), frontier and fins (dorsal and anal fins and border between fins and fish body), urogenital orifice, and body (which includes the caudal fin and peduncle and excludes the previously mentioned areas). Flounders with only healed ulcers at the time of examination were not considered as infected.

### 4.2.4 | Data analysis

Disease prevalence (number of infected fish) and mean number of ulcers per infected fish were calculated for all the examined fish for each stock, life stage and sex (adult vs. juvenile and female vs. male, respectively) and experimental treatment. Differences in prevalence were assessed with Chi-square tests.

Differences in the mean number of ulcers were assessed with non-parametric Kruskal-Wallis test. Post-hoc comparisons were made when significant differences were observed. Statistical significance for all analyses was set at $\alpha$=0.05, and analyses were performed with SPSS statistical package (SPSS v24.0).

## 4.3 | Results

### 4.3.1 | Experimental fish

During the quarantine period (57 days) of stock 1, 54 flounder died, of those 19 died naturally, and 35 were euthanized due to fish condition and welfare concerns because it was clear that most of the stock was infected and that isolation of the affected individuals (n=23) would not prevent further contamination of the rest of the stock.

With stock 2 there was a total of 4 natural deaths during the quarantine period and only 4 were euthanised.

During the quarantine period of stock 3, 4 flounder died and 10 were euthanised.

The daily mortality rate was calculated including both natural and induced (euthanasia) deaths since fish which were euthanised were clearly deteriorating and recovery seemed unlikely. Mortality rate during the quarantine periods was highest for stock 3 and lowest for stock 2 (stock 1: 1,32% $day^{-1}$; stock 2: 0,53% $day^{-1}$ and stock 3: 2,03% $day^{-1}$).

### 4.3.2 | Microbiological cultures

Preliminary results from the cultures showed that there was no septicaemia in either of the fish (given the negative cultures with the material collected from the kidney). The cultures with material from the skin lesions had different microbial growth, while the cultures made from fish with open lesions showed growth of *Vibrio* spp. and *Pseudomonas* spp.; cultures grown from a healing ulcer only exhibited growth of *Pseudomonas* spp.. The preliminary results from the water sample also exhibited the presence of *Vibrio* spp..

### 4.3.3 | Examination

A total of 110 flounder were examined (size range: 27.01±3.66 cm, 197.6±88.5 g): 45 were identified as females, 41 as males and 24 as juveniles. Flounder examination resulted in registering a disease prevalence of 80%, with a mean ulcer rate of 2.92 ± 2.31 per fish. The maximum number of ulcers per fish was 14 but this was only observed in one individual. Healed ulcers were also observed but

mainly on juvenile fish. In 32% of the fish the parasitic copepod *Acanthochondria cornuta* was found on the pectoral fins (figure 4.1).

***Figure 4.1*** *– Flounder with copepods (Acanthochondria cornuta) attached to the ventral pectoral fin.*

The internal observation of the flounder showed: 2 flounder with abnormal digestive tract and 18 with abnormal liver, i.e. with a different texture and/or colouration than the usually observed in healthy flounder; 10 with a distended abdomen due to the accumulation of fluid in the peritoneal cavity; and 2 emaciated flounder. Internal parasites were seen in the digestive tract of 12 flounder (10.9%) (figure 4.2) and in the liver of 2 individuals.

***Figure 4.2*** *– Parasitized digestive tract.*

Ulcers were typically round dark reddish open wounds, some superficial and others penetrating deeper into the musculature, and with a few irregularly shaped being registered. Examples of the observed ulcers in the 4 examined areas can be seen in figure 4.3.

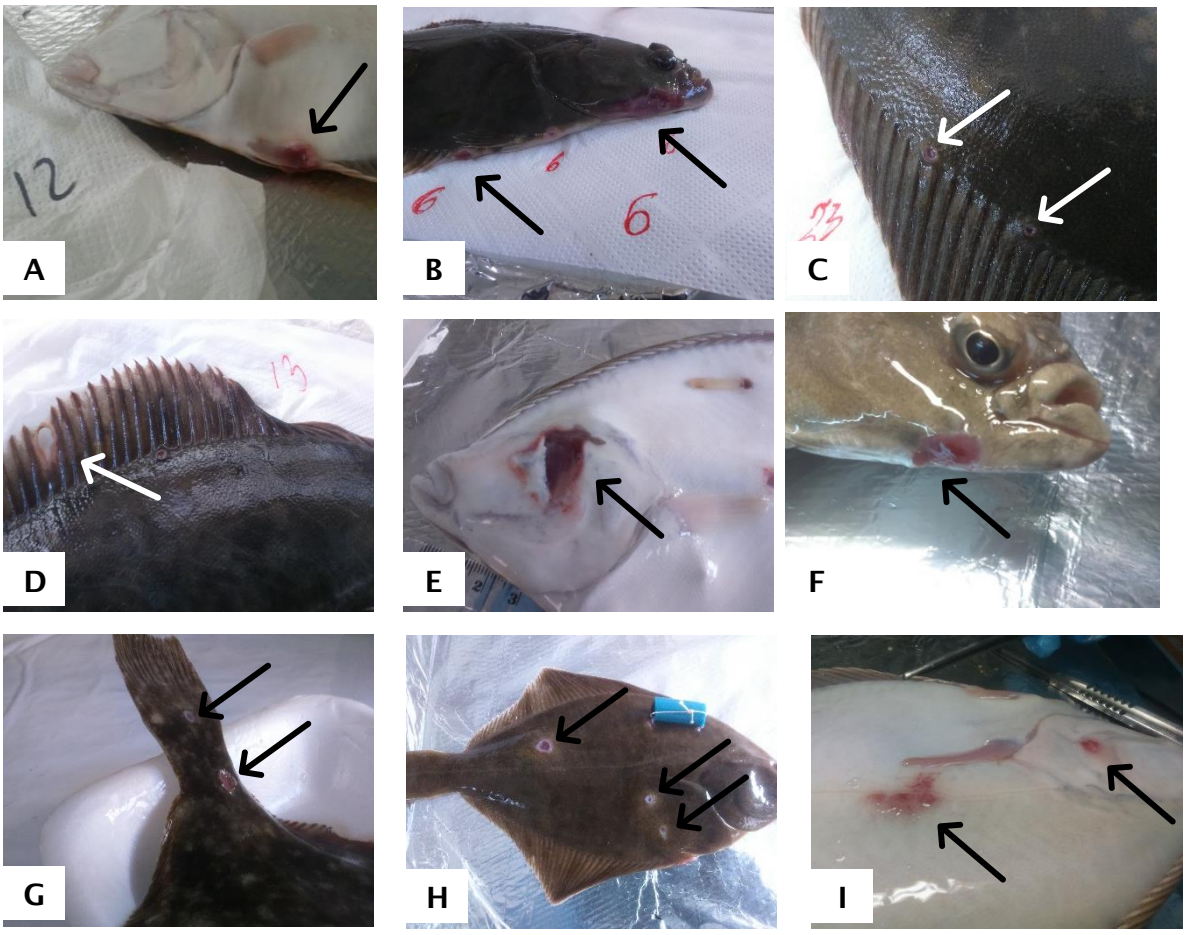


***Figure 4.3 – Photos of P. flesus with skin ulcers.***

***(A) –*** *Ulceration of the urogenital orifice;* ***(B) –*** *Ulcers on the mandible/jaw and on the urogenital orifice;* ***(C) –*** *Two ulcers in the frontier area (between the dorsal fin and the fish body);* ***(D) –*** *Ulcer in the dorsal fin and in the frontier;* ***(E) –*** *Ulcer in the head on the uneyed side;* ***(F) –*** *Ulcer of the mandible/jaw;* ***(G) –*** *Two ulcers in the caudal peduncle on the eyed side;* ***(H) –*** *Three ulcers on the eyed side of the fish body;* ***(I) –*** *Ulcers on the uneyed side. Black and white arrows point towards the ulcers.*

The general distribution in percentage of the 257 recorded ulcers and of the infected flounder across the 4 examined areas can be seen in table 4.1. The area most affected by ulcers was clearly the body with 69% of the registered ulcers and the remaining areas being affected with less than 25% of the ulcers. When assessing the areas in which flounder were more affected it is clear that most flounder developed ulcers in the body area (63%) but the head was also an area where most flounder developed ulcers (56%).

***Table 4.1 – Number (N) and distribution (%) of the ulcers and of the infected flounder across the 4 examined areas.***

| | N | Urogenital orifice | Fins and Frontier | Head | Body |
|---|---|---|---|---|---|
| Ulcers | 257 | 14.0% | 24.9% | 22.6% | 68.9% |
| Flounder | 88 | 40.9% | 42.0% | 55.7% | 63.3% |

In table 4.2 the prevalence of skin ulcers, according to fish stock (date of capture), life stage and sex (adult vs juvenile and female vs male), and according to treatment (flounder used in tagging experiments, see chapter 3) is shown. The prevalence of ulcers was significantly different among stocks (Pearson Chi-square; $\chi^2$=11.724, df=2, p<0.05) with the prevalence being significantly higher in stock 1 (92.6%) than in the other two stocks (Post Hoc; $Stock_{1,2}$: p<0.05, $Stock_{1,3}$: p<0.05, and $Stock_{2,3}$: p=0.3992). Differences in the number of ulcers per infected fish were only significant between stock 1 and 3 with stock 1 having more ulcers (3.32 ± 2.01) (KW; $\chi^2$=12.444, df=2, p<0.05; Post Hoc; $Stock_{1,2}$: p=0.065, $Stock_{1,3}$: p<0.05, and $Stock_{2,3}$: p=0.935). According to life stage no significant differences were observed in the prevalence of the ulcers between adult (82.6%) and juvenile (70.8%) flounder (Fisher's exact test; p=0.249), however adult fish developed significantly more ulcers (3.04 ± 2.08) than juvenile fish (2.41 ± 3.10) (KW; $\chi^2$=4.369, df=1, p<0.05). In terms of sex there were no significant differences between the prevalence in females (80.0%) and males (85.4%) (Yate's correction; $\chi^2$=0.137, df=1, p=0.711) or in the number of ulcers (KW; $\chi^2$=0.006, df=1, p=0.939). Among treatment groups there were also no significant differences in prevalence (Fisher's exact test; p=0.205) or number of ulcers (KW; $\chi^2$=4.162, df=3, p=0.245). The average number of ulcers in the T-bar treatment group was however skewed by a single specimen which developed 7 small ulcers on the eyed side.

***Table 4.2 – Number (n) and percentage (%) of all examined and number (n) and prevalence (%) of infected flounder and average number (mean ± SD) of ulcers observed per infected individual; of flounders according to stock, life stage and sex and according to experimental treatment.***

| | | Examined | | Prevalence | | Number of ulcers in infected fish |
|---|---|---|---|---|---|---|
| | | n | % | n | % | Mean ± SD |
| Total | | 110 | 100 | 88 | 80 | 2.92 ± 2.31 |
| Stock | 1 | 54 | 49.1 | 50 | 92.6 | 3.32 ± 2.01 |
| | 2 | 30 | 27.3 | 22 | 73.3 | 2.50 ± 2.09 |
| | 3 | 26 | 23.6 | 16 | 61.5 | 2.25 ± 3.19 |
| Life stage and Sex | Adult | 86 | 78.2 | 71 | 82.6 | 3.04 ± 2.08 |
| | Female | 45 | 40.9 | 36 | 80.0 | 3.17 ± 2.36 |
| | Male | 41 | 37.3 | 35 | 85.4 | 2.91 ± 1.77 |
| | Juvenile | 24 | 21.8 | 17 | 70.8 | 2.41 ± 3.10 |
| Treatment | Control | 16 | 14.5 | 14 | 87.5 | 2.07 ± 1.59 |
| | External | 22 | 20 | 14 | 63.6 | 2.57 ± 2.21 |
| | Implant | 10 | 9.1 | 6 | 60.0 | 1.17 ± 0.41 |
| | T-bar | 8 | 7.3 | 4 | 50.0 | 4.75 ± 6.18 |

Table 4.3 shows the total number (n) of infected fish and of ulcers; and the distribution (%) of the ulcers according to life stage and sex across the 4 examined areas. Generally, ulcers were mainly located in the fish body (68.9%). While the body and the head were the most affected areas in female flounder, in males it was the body and fins and frontier areas. In the adult flounder the fins and frontier and the head were similarly affected areas (24.1% and 24.5%, respectively), but in juvenile flounder there was a clear difference between these two areas: with the fins and frontier with 29.3% of the ulcers and the head with only 12.2%.

***Table 4.3 – Number (n) of infected flounder and of observed ulcers according to sex and life stage; and distribution (%) of the observed ulcers between/across the 4 examined areas. F = infected flounder; U = ulcer.***

| | n | | Urogenital orifice (%) | Fins and Frontier (%) | Head (%) | Body (%) |
|---|---|---|---|---|---|---|
| | F | U | U | U | U | U |
| Adult | 71 | 216 | 15.3 | 24.1 | 24.5 | 36.1 |
| Female | 36 | 114 | 14.0 | 18.4 | 26.3 | 41.2 |
| Male | 35 | 102 | 16.7 | 30.4 | 22.5 | 30.4 |
| Juvenile | 17 | 41 | 7.3 | 29.3 | 12.2 | 51.2 |
| Examined | 88 | 257 | 14.0 | 24.9 | 22.6 | 68.9 |

Figures 4.4 and 4.5 show the average number of ulcers across the four examined areas according to life stage and sex. Additionally, the number of ulcers in the body area is further explored by splitting this area in two: the eyed and the uneyed side.

The body is clearly the area where more ulcers developed; however, splitting this area into eyed and uneyed sides does not seem to have resulted in observing more ulcers on either side according to either life stage or sex. What is more evident is that juvenile fish developed much fewer ulcers in the urogenital orifice and head than adult flounders. Differences between sexes were subtler, nonetheless female flounder seem to have developed more ulcers in the body area than males, while male flounder seem to have developed more ulcers in the fins and frontier area than female flounder.

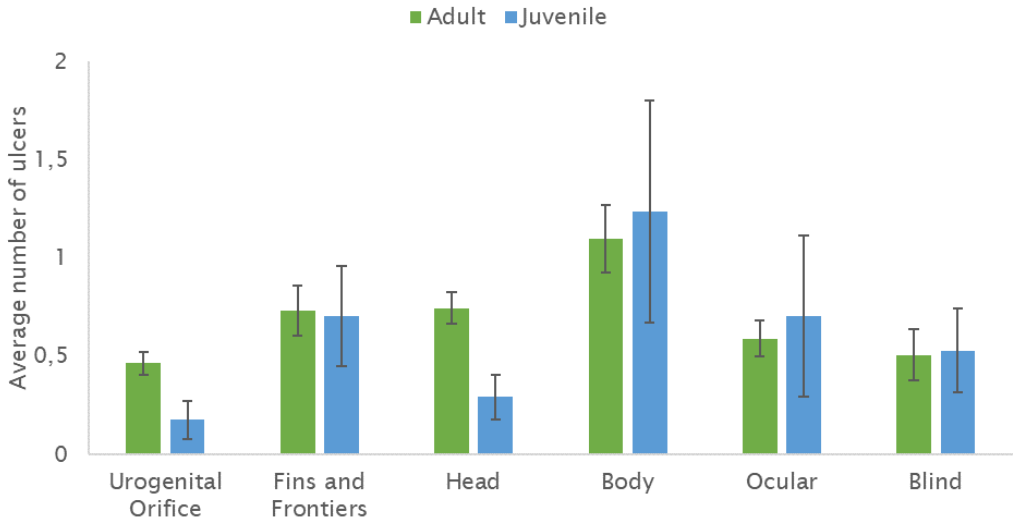


***Figure 4.4*** *– Average number of ulcers across the four examined areas according to life stage, adult (n=71) and juvenile (n=17). The body area was split into eyed and uneyed sides to further analyse the ulcers' distribution.*

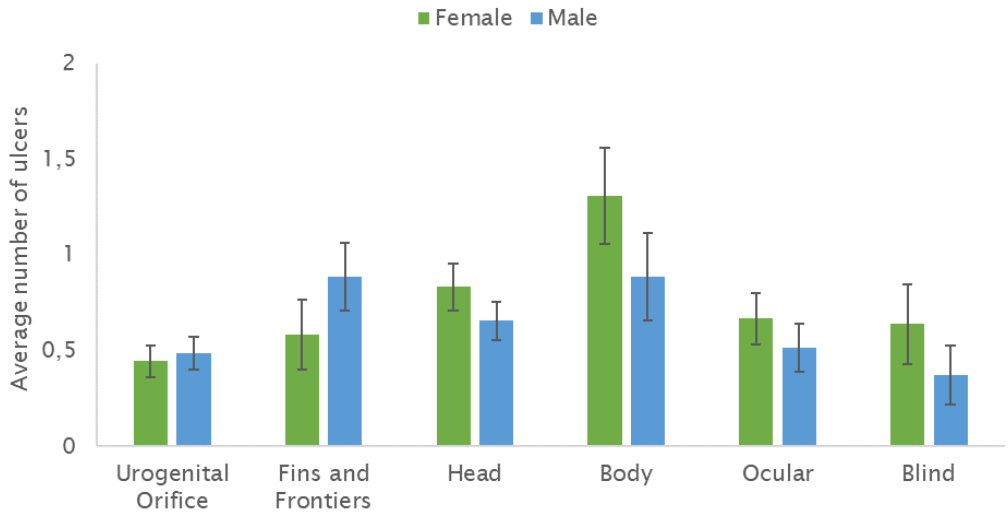


***Figure 4.5*** *– Average number of ulcers across the four examined areas according to sex, female (n=36) and male (n=35). The body area was split into eyed and uneyed sides to further analyse the ulcers' distribution.*

## 4.4 Discussion

There is increasing evidence that skin damage can be caused not only with direct contact but also indirectly due to physiological changes associated with environmental stressors (Noga, 2000). Primary skin damage can cause chronic or acute mortality (depending on the severity of the damage), increased susceptibility to diseases caused by opportunistic pathogens and slower growth (Noga, 2000). Skin is the first defence against pathogens constituting a barrier to microbial invasion and contains antimicrobial chemicals. However, when this barrier is compromised not only does it give access to infectious agents but also produces an osmotic stress that can be life threatening (Noga, 2000). During the quarantine periods the mortality rate was highest in stock 3, however the prevalence of this skin disease and the quantity of ulcers per infected fish was significantly higher in stock 1. The reasons behind this are not clear. Outbreaks are usually more severe at higher temperatures (Devesa *et al*., 1989), which was not the case with stock 1 which was kept approximately at 16.6±1.4˚C (c.f. stock 2 – 19.7±0.9˚C and stock 3 – 19.9±1.3˚C (see annexe)). Salinity also does not seem to explain the differences in prevalence as it was kept at very similar levels in all three stocks (see annexe). The most notable differences between stock 1 and stocks 2 and 3 were the date of capture, presence/absence of feeding during quarantine, and stocking density; any of these factors might have had an immunosuppressant effect on the fishes' immune systems. Fish from stock 1 were captured in January which corresponds to the reproductive season of this species in the Douro estuary. The energetic cost associated with gonadal development might help explain the higher susceptibility to this disease. Moreover, fish from stock 1 did not feed during the initial 38 days of the quarantine period, while stock 3 spent only 14 days without feeding, which might have aggravated their condition factor which is assumed to be already depressed due to the allocation of energy to reproduction (Vethaak & Jol, 1996). Also, the stocking density was higher in stock 1, with more and larger fish being kept in the same conditions as stock 2 and 3. Overcrowding is another stressful factor (Dinis, 2012) that might have increased the vulnerability of these fish. Although stock 3 also went more time, initially, without being fed than stock 2, other reasons, besides food intake, can be considered to explain why the prevalence of the skin ulcers was lowest in this stock. Stock 3 was composed mostly of juvenile flounder, which means that their condition factor was probably higher, without having dispensed additional energy in gonadal development, and adding to that the fact that most of the healed ulcers

were recorded in this stock, these fish are probably more resilient.

Ulcers examined in the present study were of the same type as those reported in previous field studies (Wiklund & Bylund, 1993; Vethaak & Jol, 1996; Drevs, 2006). However, these field studies report much lower prevalence values than those recorded in this study with captive fish. Moreover, besides the differences from laboratory and field studies, these field studies reporting on the skin ulcer disease prevalence are all with wild populations of *P. flesus* from Northern Europe while our specimens are from the Douro estuary in Southern Europe, where observations from a parallel study with wild flounder have not recorded the presence of ulcers in these fish (personal observations). Wiklund & Bylund (1993) reported a higher prevalence (5.9% to 11.1%) than those observed in previous studies with flatfish that reported values between 0% and 4%, and that were affected by sex, size, and sampling location. Lang *et al.* (1999) reported prevalence of 5.9% which was influenced by sex, size, age and salinity. Vethaak (2013) reported that the prevalence of skin ulcers between 1988 and 1996 was approximately 6.9% but values as high as 38.9% were observed in one of the sampling sites; the major contributing factor was pollution and large salinity fluctuations. Drevs (2006) observed skin ulcer prevalence of 8.2% in males during late summer and autumn and that prevalence varied seasonally. The higher values reported by Vethaak (2013) are still quite a bit lower than those reported in this study. However, while Lang *et al.* (1999) and Vethaak (2013) recorded higher prevalence values due to large fluctuations in salinity, especially in areas where salinity decreased significantly (as is the case near the drainage sluices in Vethaak, 2013), in our study after the initial change in salinity between the site of capture and the captive conditions the salinity values remained fairly constant, as previously mentioned, and higher than those registered in the field. While no statistically significant difference was observed in prevalence between life stage and sex, the number of ulcers was significantly higher in adults than in juveniles, reinforcing the idea of a higher resilience by juveniles to this disease. Moreover, it has been hypothesized that the occurrence of skin ulcers is somehow correlated with the sexual maturation of the fish, even if there still is no definite proof as to the factor related to the maturation process that induces or makes the fish susceptible to this disease (Wiklund & Bylund, 1993).This possibility seems to partially explain our observations since in our study there is an overlap on fish size and the distinction between adult and juvenile flounder was made based on gonadal development. The fact that adults, in our study, develop significantly more ulcers can be

evidence of a correlation between sexual maturation and the intensity of the skin ulcer disease. The prevalence was higher in male flounder however infected females developed more ulcers than infected males. These non-significant results are in contrast with the remaining literature (Wiklund & Bylund, 1993; Lang *et al.*, 1999; Drevs, 2006) where an obvious effect of sex has been observed, with males being more susceptible.

Diseases are usually caused by some stress that affects the fishes' natural defences and immune system. It seems likely that sub-optimal captivity conditions might have resulted in a higher vulnerability of flounder to infectious agents naturally present in the system. Optimizing these conditions might prevent its reappearance in future experiments.

# Chapter 5 | General discussion

## 5.1 | Captivity and tagging

A tagging methodology was successfully developed and tested in a laboratory setting to allow the tracking of flounder in the field.

Aspects related to fish handling and manipulation to perform tagging procedures in laboratory conditions were operationalized and optimized during the course of this study. Analgesia and sedation of the study species was standardized for experiments performed at high temperatures (19 to 20˚C) and with fish ranging from 70g to 300g. It is important to note, however, that concentrations and times employed in these experiments should not be extrapolated for different environmental conditions, fish sizes or even other species. Tagging procedures became faster and more expedient from experiment 1 to experiment 2, decreasing the handling time which is important for reducing stress of the tagged individuals. However, maintaining flounder in captivity proved to be extremely challenging with unexpected high mortalities in the quarantine phase. It was not possible to maintain optimal water quality parameters because neither reduction of stocking density or increases in biofilters caused substantial improvements in the water quality.

In terms of induction times for flounder anaesthesia our initial assessment in Chapter 2 might be a little conservative. During the tagging trials, it was observed that fish which remained only two to two and a half minutes in the induction bath reached the same anaesthesia stage which could also be maintained during the whole tagging procedure. Moreover, besides the initial lack of negative effects after analgesics administration, it appears that the use of analgesics might actually be beneficial, as positive control groups had higher survival rates than negative control groups. The reason behind this increased survivorship is not clear, but maybe it can be attributed to a decreased stress response to manipulation after analgesics administration.

A major hindrance during this study was the elevated mortality, during the quarantine and experimental periods. The high mortality of the control groups, 60% in experiment 1 and 33.3% in experiment 2, lead us to think that the negative effects observed at the end of the experiments were probably exacerbated by a factor other than the tagging procedure itself. Mortality was especially high in the case of implanted tags. The use of antibiotics in tagging procedures is a controversial subject (Pursche *et al.*, 2014), nevertheless it is commonly used

especially in intracoelomic procedures. In this study it was decided not to administer antibiotics mainly because using them in a single dosage has, of yet, not been proved to be effective and could lead to the development of antibiotic resistance in bacteria and changes in the immune system of the fish (Mulcahy, 2011). Besides, antibiotics should not be used as substitute of aseptic technique, the perioperative use of antibiotics can be obviated if due care is taken to prevent contamination of the incision, transmitter or instruments (Mulcahy, 2003). Since neither the use of aseptic techniques or of antibiotics have been proved to increase the success of tagging procedures or improve healing (Cooke *et al.*, 2011), the use of aseptic techniques seemed advantageous when compared to the drawbacks associated with the use of antibiotics.

The external procedure had the least negative impacts on flounder. It does not seem to have affected the fish's behaviour as feeding behaviour and overall activity were not inhibited after the tagging procedures. However, impacts on survival and physiology (condition and growth) although negative, were not completely clear because other factors, namely the stress associated with captivity might have interfered with the effects caused by the tagging procedures. Skin ulcer disease which affected 80% of the experimental fish might have been the major contributing factor for the negative effects on survival and growth instead of the tagging procedures. Fish with damaged epithelia have to spend more energy on repairing wounds and on osmoregulation which might lead to slower growth, increased susceptibility to diseases and chronic mortality. Moreover, in coho salmon (*Oncorhynchus kisutch*) it has been shown that lesions of as little as 10% of the surface can result in acute mortalities (Noga, 2000). Replication of the experiments with healthy fish would be necessary to accurately assess the effects of the tagging procedures on fish.

Tag loss was only observed when marking fish with the plastic tags and was very likely caused by bad placement. Fish marked with acoustic tags did not lose, expel or were they observed actively trying to remove the tags.

A field experiment should help clarify the results gathered in the laboratory experiments and help assess other effects that can only be surmised in the field. For instance, risk of entanglement and other natural stressors, such as predation risk, pathologies, and density, are very challenging to accurately replicate in captivity.

## 5.2 | Field recommendations

Based on the laboratory experiments some recommendations to improve tagging procedures and associated handling and manipulation could be established.

To minimise stress during capture fish trawls should be shorter, and different capture methods (e.g. gillnets) should be employed. Collection of less fish at a time would decrease the chances of fish compressing each other during entrapment in the net. Fish should also be promptly accommodated in coolers with aerated water. Experimental fish should be marked in small batches, i.e. the quantity of fish captured and waiting to be marked should also be small to decrease the time they are kept in confinement and thus under stress.

Anaesthetics concentrations should also be re-evaluated, with a small fish sample, if the field experiment is to be performed under different environmental conditions or with fish with a different size range than the ones tested here. Anaesthetic baths, induction and maintenance, should be made anew when it starts to weaken as reinforcement of these baths with additional dosages can make them too concentrated.

Some improvements to the external mounting procedure could also be considered. Using needles of a slightly larger gauge can decrease the time spent threading the line through the needles which will reduce handling time and time spent under anaesthesia. The use of the thinner tubing on fish's uneyed side can also be reconsidered, it is not clear if it is necessary to protect the line, but it seems likely that the use of this tube is either impairing healing or causing the inflammation on the uneyed side.

Evaluating tagging effects and procedures is a crucial step before any telemetry study; only then is it possible to know if the data being collected accurately represent the untagged population. Even for well-studied species there is a surprising paucity of data on the effects of tagging. Even though these effects might be species specific, more data from different species would be helpful to create standard operating procedures, or at least have a better idea of which procedures are more or less adequate for some species. A particular concern should be the welfare of the research animals involved.

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| Annexe – Water quality

1 | Stock 1

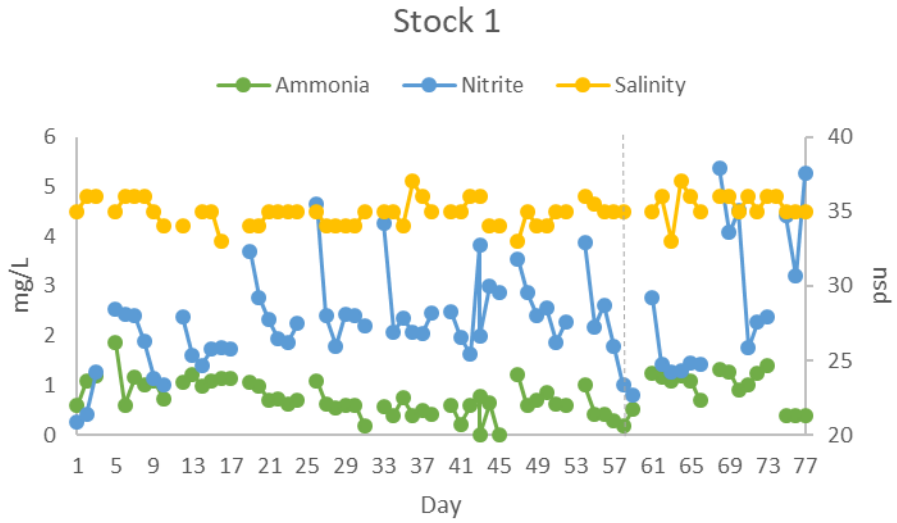



***Figure A.1.1*** *– Ammonia, nitrite and salinity values during the entire captivity period. The vertical line on day 58 signals the end of the quarantine period and the beginning of the isolation period.*

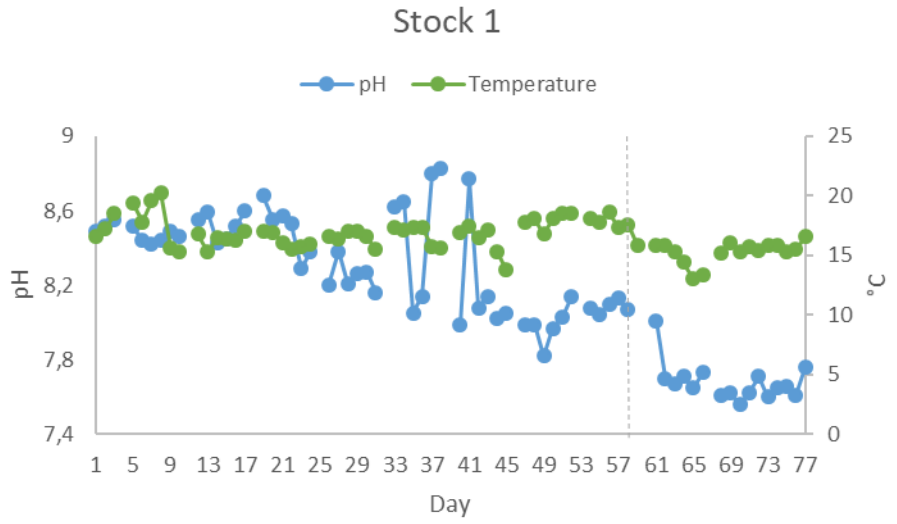
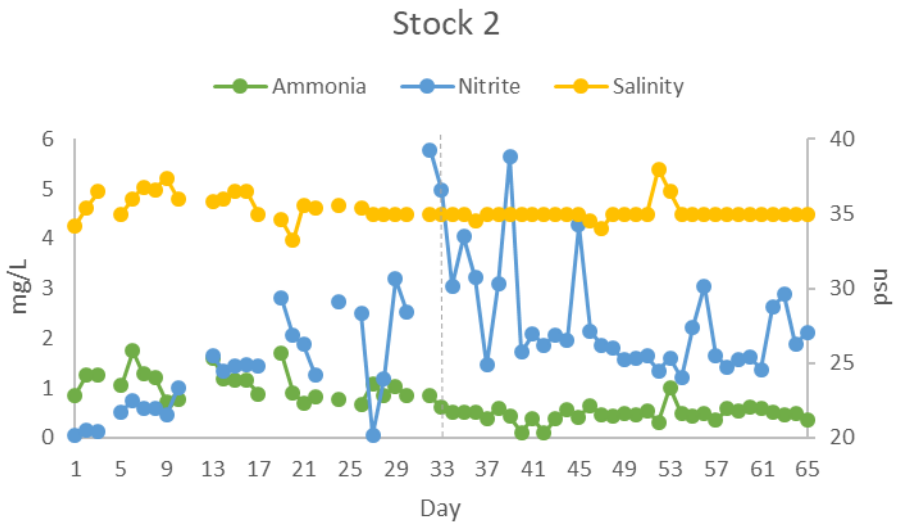



***Figure A.1.2*** *– pH and temperature values during the entire captivity period. The vertical line on day 58 signals the end of the quarantine period and the beginning of the isolation period.*

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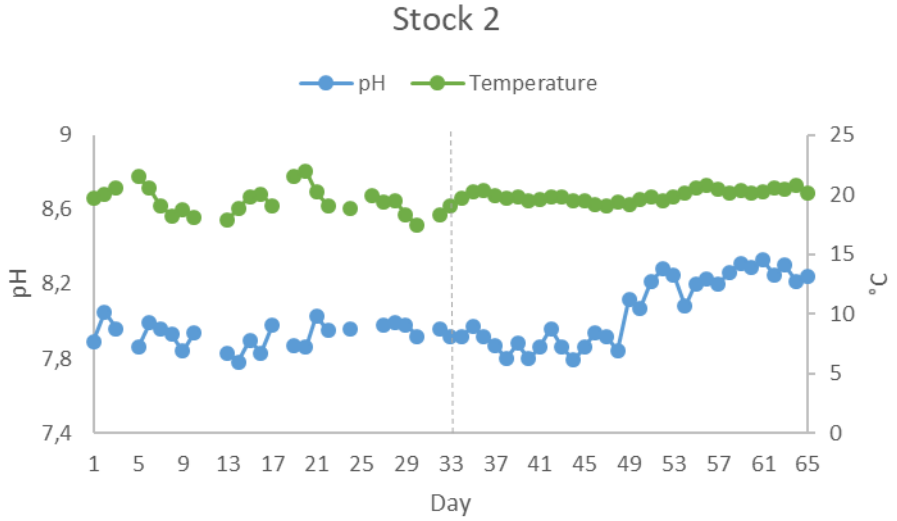


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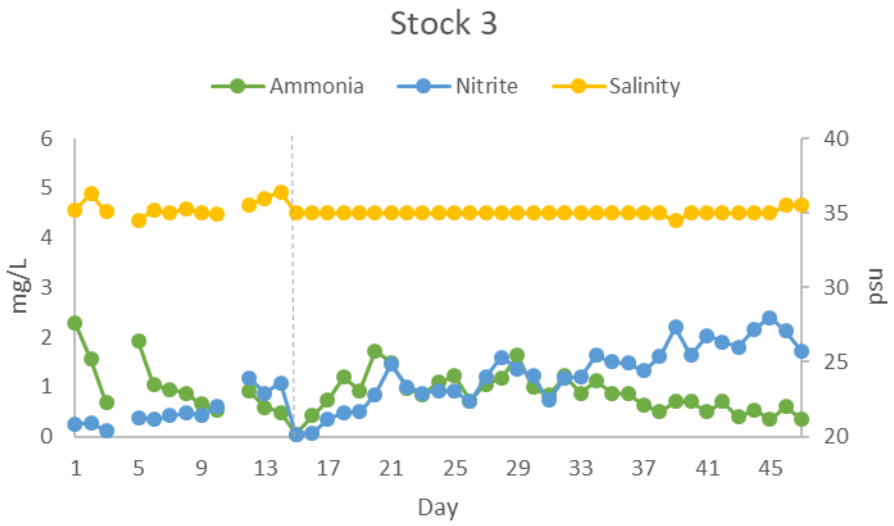


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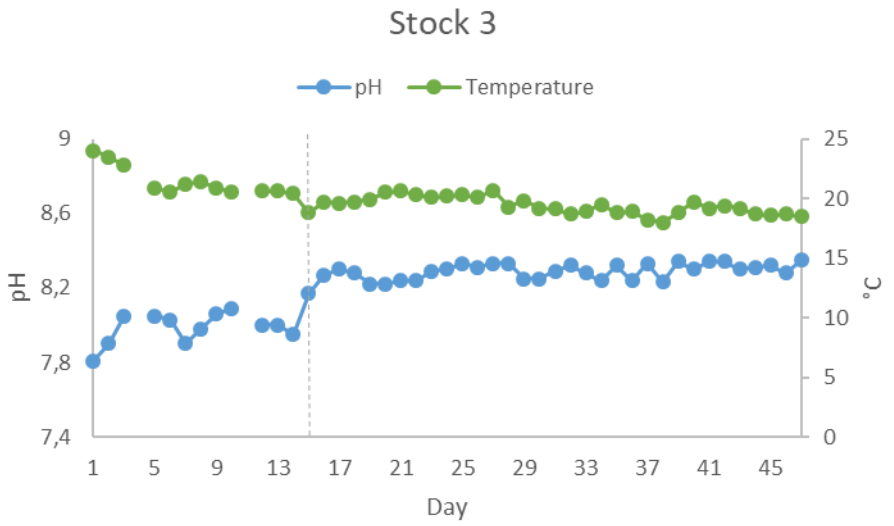


***Figure A.3.2*** *– pH and temperature values during the entire captivity period. The vertical line on day 15 signals the end of the quarantine period and the beginning of the experimental period.*